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14. ABSTRACT

The purpose of this project is to investigate the relationship between phasic dopaminergic signaling and behavior in an animal model of Parkinson's disease. The overall hypothesis is that, in rats with partial dopamine lesions mimicking the preclinical phase of Parkinson's disease, deficits in phasic dopaminergic signaling are associated with behavioral deficits. Phasic dopaminergic signaling is characterized by chemical microsensors measuring dopamine, and electrophysiology is used to monitor the effect of dopamine on target cells. Behavioral tests are also developed and assessed to identify deficits that occur during partial dopamine depletion. Highlights of Year 2 include the first ever recording of electrically evoked dopamine levels, spontaneous dopamine transients and drug-induced dopamine transients in a freely moving, lesioned rat and the first recording of voltammetry and electrophysiology at the same microsensor in an anesthetized, lesion rat and in a freely moving, non-lesioned rat. Two studies evaluating sensorimotor tests are completed, and manuscripts are in preparation. Other behavioral tests and a new lesion procedure, extending those previously proposed, are in development.

15. SUBJECT TERMS
Parkinson's disease, dopamine, compensation, phasic signaling, microsensors, electrophysiology behavior

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Introduction

This progress report details accomplishments during the second year of USAMRMC 03281055. The overall goal of this award is to characterize phasic dopaminergic signaling in the 6-hydroxydopamine-lesioned rat model of Parkinson's disease. Dopaminergic neurons employ two modes of communication, tonic and phasic. Tonic signaling provides an ambient, steady-state level of extracellular dopamine, whereas phasic signaling results in a transient increase (i.e., a short-lived concentration spike). While tonic dopamine levels are known to be normalized via compensatory mechanisms during the preclinical phase of Parkinson's disease, our overall hypothesis is that phasic dopamine transients decrease in amplitude, suggesting that compensation, if present, is downstream. We also hypothesize that a decrease in the size of dopamine transients results in behavioral deficits, both in experimental animals and also during the so called preclinical phase of Parkinson's disease.

As with the first year, there are aspects of the project that are on schedule, ahead of schedule and behind schedule. Please refer to the *Statement of Work* below for year to year goals. In the first year, characterization of phasic dopaminergic signaling by chemical microsensors in anesthetized animals was completed. Instead of performing two asymmetry tests assessing sensorimotor function, five tests were performed altogether. These included two tests originally proposed, cylinder- and vibrissae-evoked paw placement, and three others, engagement, disengagement and passive-initiation tests. The passive-initiation test is novel and was developed by Co-PI Schallert. Continuing development of instrumentation for combined voltammetry and electrophysiology precluded application of this technique during the first year. In year two, assessment of the five asymmetry tests for sensorimotor function was completed. Work towards developing additional tests was also performed. Characterization of phasic dopaminergic signaling by chemical microsensors in freely moving animals and measurements using both voltammetry and electrophysiology in anesthetized animals at the same electrode were initiated. Work establishing a behavioral activation test was not begun. However, in collaboration with another investigator, combined voltammetry and electrophysiology in freely moving animals, a goal for the third year, was started. In addition, work developing a new procedure for generating dopaminergic lesions in animals to be used for voltammetric and/or electrophysiological measurements without anesthesia was begun. This procedure, which will increase throughput and provide intra-animal comparisons, was not proposed in the original project.

Overall, the second year was highlighted by several firsts for research using freely moving, lesioned animals, including the measurement of electrically evoked dopamine levels, endogenous phasic dopamine transients, and drug-induced dopamine transients. Dopamine and the electrophysiological response to dopamine were also measured in the anesthetized, lesioned animal and in the freely moving, non-lesioned animal at the same electrode for the first time. Initiated in year two, these experiments will continue into year three along with work related to the goals for that year.

Body

Statement of Work (verbatim from proposal)

Year 1

Phasic dopaminergic signaling will be characterized in anesthetized animals using real-time chemical microsensors.

Two asymmetry tests assessing sensorimotor function will be performed on each of the animals above.

The technique of quasi-simultaneous voltammetry and electrophysiology will be established at Illinois State University.

Quasi-simultaneous voltammetry and electrophysiology will be used to assess adaptation of striatal target cells to phasic dopaminergic signaling in anesthetized animals.

Year 2

Phasic dopaminergic signaling will be characterized in freely moving animals using real-time chemical microsensors.

A new test for behavioral activation will be established and used to assess this behavior in each of the animals above.

Continuation of using quasi-simultaneous voltammetry and electrophysiology to assess adaptation of striatal target cells to phasic dopaminergic signaling in anesthetized animals.

Year 3

Phasic dopaminergic signaling will be characterized by real-time chemical microsensors in freely moving animals during novel exposure to a cohort.

A new test for approach behavior will be established and used to assess this behavior in the animals above.

Quasi-simultaneous voltammetry and electrophysiology will be used to assess adaptation of striatal target cells to phasic dopaminergic signaling in freely moving animals.

Asymmetry tests assessing sensorimotor function

Initiated in the first year, a study comparing five asymmetry tests assessing sensorimotor function was completed. These tests are limb-use for vertical-lateral weight shifting in a cylindrical enclosure, vibrissae-evoked paw placement, attention-related motor function (engagement, disengagement) and capacity to regain center of gravity (model of a standard "regain of balance" test in Parkinson's disease). The goal was to identify behavior deficits that emerge following partial lesions of nigrostriatal dopaminergic neurons mimicking the preclinical phase of Parkinson's disease. The rationale was that the hypothesized decrease in the amplitude of phasic dopamine transients following a preclinical level of dopamine denervation is associated with behavioral deficits. This study is the most comprehensive to date for these tests. Only the limb-use cylinder test was sensitive to partial dopamine denervation. Details of this study, first reported last year at the Annual Meeting for the Society of Neuroscience (Abstract # 676.5, Mithyantha et al., 2004), are found in the 2005 M.S. thesis of Jahnavi Mithyantha (Appendix I). A manuscript is in preparation. A review chapter on sensorimotor function tests has also been published (Schallert and Woodlee, et al. 2005, Appendix II).

Also reported last year at the Annual Meeting for the Society of Neuroscience (Abstract # 562.16, Woodlee et al., 2004) was that the regain of balance test revealed not only a deficit in the

limb opposite the dopamine depletion, but also compensation in the intact side of unilaterally lesioned animals. In this test, the experimenter restrains all but one forelimb and then slowly moves the rat's body forward on a flat surface, which alters center of gravity. The capacity to respond to this imposed shift in center of gravity was reflected in the amount of forward movement needed to trigger a catchup step in the planted limb. In contrast to what has been shown previously by Schallert and others using rapid forward or lateral movement, slow movement stepping remains intact even after severe dopamine depletion, but the response is very much delayed, as it is in patients with Parkinson's disease. This delay in reactivity to shifts in center of gravity is one of the biggest dangers to patients because it leads to falling. We found that the unimpaired forelimb of lesioned animals requires a smaller weight shift to elicit such a step than does either limb of non-lesioned controls, suggesting a reorganization of circuitry in the intact hemisphere. This study is now complete, and a manuscript is in preparation.

We continued to develop other sensorimotor tests that would be suitable for correlating with phasic dopamine transients. One test under development is a lateral-movement vibrissae-evoked placing test in which the distance that the forelimb reaches laterally onto a surface is quantified. Preliminary work suggests that this lateral distance, or range of motion, is sensitive to partial denervation in the 6-hydroxydopamine-lesioned rat and MPTP-treated mouse. Figure 1 shows preliminary data from the rat for unilateral and bilateral lesions. All animals placed with the standard vibrissae-evoked placement test, verifying that lesions were not severe.

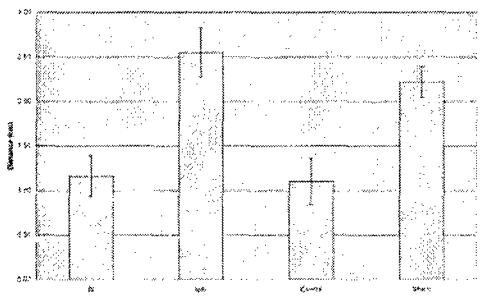

Figure 1. Lateral-movement Placing Test.

Another test that we are working on is an odor recognition memory test. The rationale is that olfactory deficits occur early in Parkinson's disease (Tissingh et al., 2001; Berendse et al., 2001). Very preliminary data indicate a deficit after administration of 6-hydroxydopamine into the medial forebrain bundle, suggesting that non-olfactory bulb dopamine depletion may be a factor (data not shown). The test is a novel adaptation of odor detection tests and involves social odor recognition, discrimination, and memory. Rats with bilateral partial dopamine depletion appear to be able to discriminate familiar from novel odors, but fail to retain memory for the novel odor overnight. Unlike normal animals, which always spend more time exploring wooden beads laced with the more novel of two odors, rats with moderate to severe dopamine depletion do not appear to distinguish between a completely novel odor and a recently familiar but not completely novel odor. As in the early subclinical stages of Parkinson's disease, in which there is no anosmia per se but rather a complex inability to distinguish among a range of odors, this odor recognition deficit may be sensitive to low levels of dopaminergic terminal loss. Because the deficit is observed without toxin exposure in the olfactory bulb, this may help explain why in Parkinson's disease one does not find a loss of tyrosine hydroxylase in the olfactory bulb.

Cross midline placing tests were also developed and these results will be presented at the 2005 Annual Conference for the Society for Neuroscience (Woodlee et al., abstract, Appendix III). This test indicated that in the unilateral dopamine depleted rat, sensory input from the impaired

side of the body into the dopamine depleted hemisphere had connected access to motor programs for limb placing on the non-impaired side. This suggests that the impairment at least in placing is not sensory but is primarily one of movement initiation. In contrast, sensory input to the vibrissae on the non-impaired side of the body could not initiate placing of the forelimb on the impaired side (i.e., akinesia). Recently we found that after partial dopamine depletion, recovery from the latter deficit occurred, such that sensory input to the intact hemisphere from the vibrissae on the non-impaired side could initiate placing of the forelimb on the impaired side. Thus, cross-midline sensorimotor integration, but not within-hemisphere sensorimotor integration, was restored.

Work establishing range of motion in a surface reaching test and odor recognition tests will continue in the third year, along with development of a behavioral activation test, originally proposed for the second year (see *Statement of Work*).

Dopamine measurements in freely moving, lesioned animals

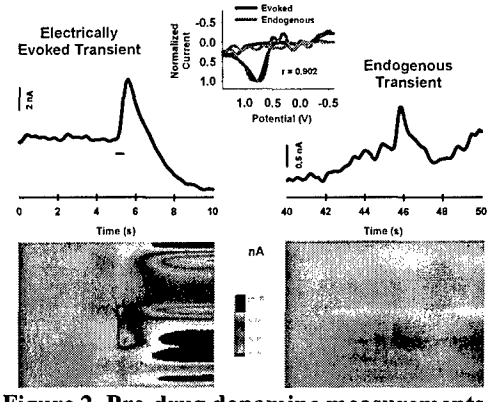

Figure 2. Pre-drug dopamine measurements in a freely moving, lesioned rat.

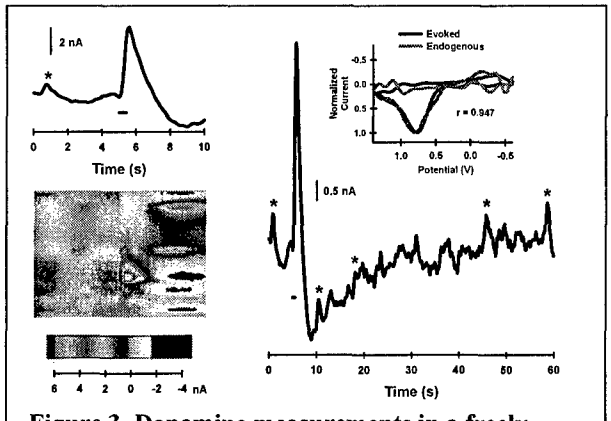

Figure 3. Dopamine measurements in a freely moving, lesioned rat following WIN55,212-2

One highlight of year two was establishing the real-time measurement of dopamine in freely moving, lesioned animals, which has not previously been performed. Similar measurements have been reported in non-lesioned animals (Robinson et al., 2001; Robinson et al., 2002; Roitman et al., 2004; Cheer et al., 2004). Figure 2 shows an electrically evoked signal at about 5 s (middle trace, left panel) and, approximately 40 s later, a spontaneous (i.e., non-electrically evoked) concentration spike (middle trace, right panel) collected in a freely moving, lesioned rat. Stimulation was applied to the medial forebrain bundle to evoke the trace shown in the left panel. This type of stimulation is well established to elicit dopamine in the striatum of freely moving rats (Garris et al., 1997; Garris et al., 1999; Garris et al., 2003), the location of the voltammetric microsensor. The INSET at the top overlays the background subtracted cyclic voltammogram obtained from electrical stimulation (black line) and the endogenous transient (red line). Voltammograms are similar (r = 0.902) and indicate that dopamine underlies the increase in both signals. Below each trace is a color plot showing all of the electrochemistry recorded. The x axis is time and is the same scale as the microsensor recording (middle trace). The y axis is applied potential and is the same scale as the x axis of the voltammogram (INSET, top). The z axis is current. The greenish dot at approximately 5 s corresponds to the increase in dopamine evoked by the electrical stimulation (middle trace, left panel) and the large, downward (i.e.,

oxidative) peak around 0.7 V in the dopamine voltammogram (black line, INSET, top). Other features after the electrical stimulation are due to changes in brain pH, which cause the trace to decrease at longer times after the stimulation. This artifact is readily removed to obtain a pure dopamine signal (Venton et al., 2003; Roitman et al., 2004). Although the spontaneous dopamine transient is smaller, there is a clear purplish dot at about 45 s (bottom, right panel), also corresponding to the dopamine signal increase (middle trace, right panel) and the dopamine voltammogram (red line, INSET, top).

Figure 3 shows a recording from the same animal but after administration of WIN55,212-2 (5 mg/kg i.p.). This cannabinoid agonist has previously been shown to increase the frequency of phasic dopamine transients in intact (i.e., non-lesioned) animals (Cheer et al., 2004). The left panel (top trace) shows an expanded view of the first 10 s of a recording collected 5 min after drug administration. In addition to the evoked signal at about 5 s, there is a smaller, spontaneous transient at about 1 s. The color plot underneath indicates that both signals are dopamine. The right panel (bottom trace) shows the same recording but over a longer time. The evoked signal is readily apparent, as are several smaller transients not associated with the electrical stimulation. The INSET above overlays the voltammogram recorded during electrical stimulation (black line) and during the last small transient in the trace at about 60 s (red line). Again, voltammograms are similar ($r = 0.947$) and indicate dopamine. The asterisk above each smaller peak identifies spontaneous transients whose voltammograms favorably compare with dopamine measured during electrical stimulation ($r > 0.75$). Five transients were recorded in this trace. The frequency of positively identified dopamine transients (using $r > 0.75$ when comparing the voltammogram to electrically evoked dopamine as the criterion) was 0.6 spikes/min during pre-drug recording and 5.7 spikes/min following WIN55,212-2, in excellent agreement with previous studies in intact animals (Robinson et al., 2002; Cheer et al., 2004). The striatal dopamine content of this animal was decreased by approximately 40% as shown by subsequent HPLC-EC analysis.

Similar measurements have been collected a different doses of WIN55,212-2 (2 and 10 mg/k i.p.) and in intact animals (data not shown), and this experiment is ongoing to increase sample size. The cannabinoid agonist is used to increase the number of spontaneous dopamine concentration spikes for analysis. The primary goal is to compare dopamine transients in lesion and non-lesioned animals in order to test the overall hypothesis that the amplitude of these concentration spikes decrease following partial dopamine denervation mimicking the preclinical phase of Parkinson's disease.

New 6-hydroxydopamine lesion procedure

While dopamine measurements in freely moving, lesioned rats are now becoming routine in our laboratory, the experiment is low throughput. To increase efficiency, we are developing a new 6-hydroxydopamine lesion procedure based on the previous work of Co-PI Schallert (Fleming et al., 2005). The design is to implant a cannula in the substantia nigra during the preparatory surgery, but administer 6-hydroxydopamine at a later time. The rationale is that microsensor measurements can be made in the same animal prior to and after neurotoxin injection, thus using the same animal as control and after lesions. It may even be possible to inject 6-hydroxydopamine at two different times, thus recording dopamine in the same animal at two

different lesion degrees, and even to administer drugs into the same cannula (e.g., glutamate agonists) to induce phasic firing.

Figures 4 and 5 show preliminary data (n = 3 to 4) developing this new lesion procedure. Figure 4 shows the cylinder test, the sensorimotor function test that is sensitive to partial dopamine denervation (Appendix I), prior to the cannula implantation surgery (Pre-Can), after the surgery (Post-Can), and after injection of 6-hydroxydopamine for different cannula lengths. The number in mm is the distance that the injection cannula protrudes from the guide cannula. While the implantation surgery did not affect asymmetry, neurotoxin administration appears to increase scores for the two longer cannulas. These lengths of cannula also caused the greatest dopamine denervation as shown in Figure 5. Saline injection (Sham) did not appear to alter asymmetry score (data not shown). The long-term plan is to implant the cannula during the preparatory surgery for voltammetry to determine if both behavior and dopamine can be monitored before and after neurotoxin injection in the same animal.

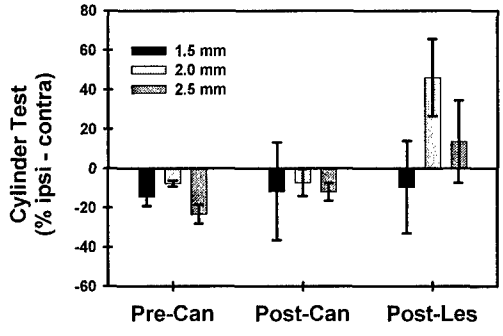

Figure 4. Forepaw asymmetry test in awake animals receiving 6-hydroxydopamine.

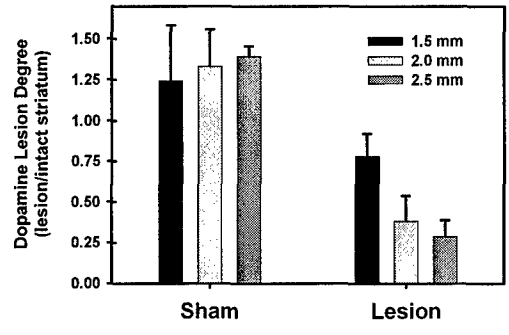

Figure 5. Dopamine tissue content after injecting 6-hydroxydopamine into awake rats.

Combined voltammetry and electrophysiology

Another highlight of year two was combined measurements of voltammetry and electrophysiology at the same carbon-fiber microelectrode. Effort was directed at establishing these measurements in anesthetized, lesioned rats, which have previously been performed in anesthetized, intact animals (Williams and Millar, 1990a; Williams and Millar, 1990b). While carbon-fiber microelectrodes are well established for their utility as electrochemical microsensors (Garris and Wightman, 1995), they are also excellent sensors for electrophysiological recording of single unit activity, an observation made almost 25 years ago (Armstrong-James et al., 1981). In fact, we found that carbon-fiber microelectrodes performed as well as or even exceeded glass microelectrodes (data not shown), considered by many the gold standard. Combined voltammetry and electrophysiological measurements have previously been performed at the same sensor either by electrically stimulating dopaminergic neurons or by iontophoretically applying dopamine (Williams and Millar, 1990a; Williams and Millar, 1990b). We will eventually do both, but opted for iontophoresis initially, because we would also like to administer dopamine antagonists to determine the selectivity of the effects.

Two configurations for coupling carbon-fiber microelectrodes to iontophoresis pipettes were examined. The easiest to construct was a "piggy-back" assembly consisting of a separate carbon-fiber recording electrode and iontophoresis multibarreled pipette. This configuration is

used presently. The other, a multibarreled pipette, with the middle barrel containing a carbon fiber for recording, may be used in the future if ready fabrication can be established. The advantage of the later is close spacing between injecting barrels and recording electrode, but this arrangement may also cause artifacts from the ejection current.

Figure 6 shows representative results collected with the piggy-back assembly. The five barrels contain glutamate (2 barrels, 200 mM), dopamine (2 barrels, 100 µM or 200 mM) and saline (1 barrel, 2 M), for current balancing to minimize ejection artifacts. All units were sensitive to glutamate, and dopamine was applied in the presence of glutamate. Frequency histograms were created with 2 s bins, and the voltammetric response is reported in concentration, based on post-calibration of the carbon-fiber microelectrode. Three types of responses were observed for striatal units after iontophoretically administered dopamine: inhibition (top panel), biphasic (short excitation followed by longer inhibition; middle panel) and no response (bottom panel). The ejection time for these recordings was 10 s, which elicited a dopamine concentration previously shown to cause inhibition (Williams and Millar, 1990b). Note that ejected dopamine concentrations were similar for the three different electrode assemblies. Results for shorter ejections are shown in Table 1 below.

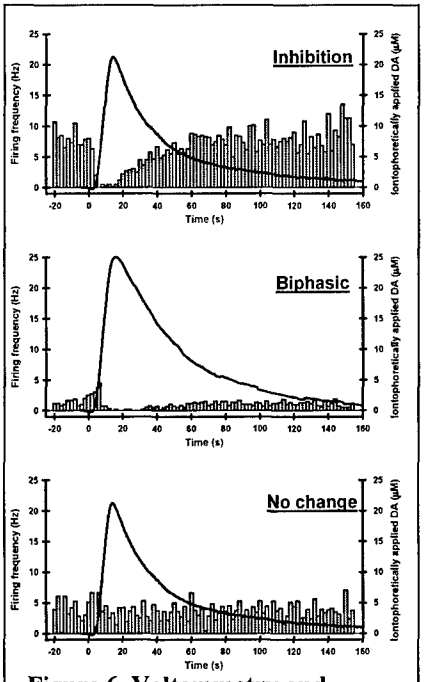

Figure 6. Voltammetry and electrophysiology in anesthetized, lesioned animals.

Table 1 shows a compilation of cells recorded in intact and 6-hydroxydopamine-lesioned rats. Responses are considered excitatory or inhibitory if firing rate changed >50% from basal firing rate. Responses are further subdivided into short latency, defined as a response occurring < 10 s after onset of DA ejections, and long latency, defined as a response occurring > 10 s after onset of DA ejections. Although preliminary, there appears to be a switch from cells transiently inhibited to cells transiently excited by dopamine in the lesioned striatum. These data and other will be presented at the 2005 Annual Meeting for the Society for Neuroscience (Sandberg et al., abstract, Appendix IV).

NAÏVE

	Ejection duration (s)							
	10 s		5s		1s		0.5s	
	Short latency	Long latency	Short latency	Long latency	Short latency	Long latency	Short latency	Long latency
Excitation	1/6	0/6	n/a	n/a	0/2	0/2	0/1	0/1
Inhibition	5/6	6/6	n/a	n/a	1/2	1/2	0/1	0/1
No change	0/6	0/6	n/a	n/a	1/2	1/2	1/1	1/1

LESION

	Ejection duration (s)							
	10 s		5s		1s		0.5s	
	Short latency	Long latency	Short latency	Long latency	Short latency	Long latency	Short latency	Long latency
Excitation	4/7	0/7	1/2	0/2	1/3	0/3	1/2	0/2
Inhibition	2/7	5/7	1/2	2/2	1/3	1/3	0/2	0/2
No change	1/7	2/7	0/2	0/2	1/3	2/3	1/2	2/2

Table 1 – Effects of iontophoretically applied dopamine on glutamate-activated single cell units in the intact and 6-hydroxydopamine-lesioned striatum of the anesthetized rat.

Efforts were also directed towards combined voltammetry and electrophysiology measurements in freely moving animals, a goal for year three. This work, a collaboration with Mark Wightman at the University of North Carolina, initially began over three years ago while PI Garris was on sabbatical and has been ongoing since. This project is now coming to fruition, and results indicate that dopamine and the electrophysiological response to dopamine can be measured at the same sensor in the unanesthetized, non-lesioned rat (Cheer et al., submitted, Appendix V). We plan to use this same approach and instrumentation in the unanesthetized, lesioned rat in years three and four.

Modeling

We continue to develop a mathematical model of extracellular dopamine regulation in the striatum during Parkinson's disease. Over the last year, our model has improved in sophistication and appears to capture experimentally established dopamine dynamics with high fidelity. This model is important for the present project, because it provides a theoretical basis for the hypothesized decrease in phasic dopamine transients during the preclinical phase. Additionally, combining theory and experiment will result in stronger papers. After completing a manuscript with the first description of the general model (Venton et al, in preparation, see rough draft in Appendix VI), we will work on two additional manuscripts. The first is a theoretical evaluation of phasic dopamine transients in Parkinson's disease. Most of these simulations have already been completed. Second, we will combine a smaller set of similar simulations with experimental data collected during the first year documenting electrically evoked dopamine transients in anesthetized, lesioned animals (see *Statement of Work* and Sandberg et al., 2004, Abstract # 953.5, Annual Meeting for the Society of Neuroscience) and a portion of the newer results collected during year two in freely moving, lesioned animals (see above). A more detailed analysis of phasic dopamine transients collected in freely moving, lesioned animals will follow once this study is completed.

Microsensor characterization

The ability to record non-stimulated phasic dopamine transients in freely moving animals, whether lesioned or not, is due to the increase in sensitivity provided by extending the waveform scan for voltammetry (Heien et al., 2003). The downside of extended scanning is that as the sensor becomes more sensitive, it loses response time, which may complicate certain types of analysis. However, the slowed response time may be dealt with using convolution techniques to restore the signal to its original dynamics (Garris and Wightman, 1995). In work that will be presented at the 2005 Annual Meeting for the Society for Neuroscience (Howes et al., abstract, Appendix VII), we show that the original signal can in fact be recaptured and that analysis for dopamine release and uptake can be performed on signals collected with the extended scan. Removing the temporal distortion will also advance other types of analysis of the phasic dopamine transients.

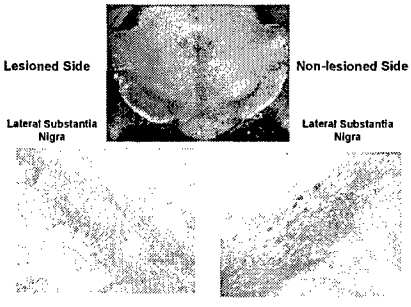

Figure 7. Tyrosine hydroxylase immunocytochemistry of the substantia nigra in a unilaterally lesioned rat.

Establishment of tyrosine hydroxylase immunocytochemistry

To augment HPLC-EC analysis of tissue dopamine content as a measure of dopamine lesion degree, we established immunocytochemistry for tyrosine hydroxylase. The top panel in Figure 7 shows a slice taken from the midbrain. The substantia nigra is bilaterally stained (brown). The bottom panels show an expanded view of the lateral substantia nigra of the lesioned (left) and intact (right) side. Another future application of immunocytochemistry is to stain the striatum in order to determine both lesion degree and microsensor placement.

Key Research Accomplishments

- Completion of a study assessing five sensorimotor tests across the entire lesion range of preclinical and symptomatic Parkinson's disease, the most comprehensive study to date describing these tests.
- Completion of a study identifying contralateral compensation using the passive-initiation test, a novel sensorimotor assessment.
- First recorded measurement of electrically evoked dopamine release in a freely moving, lesioned rat.
- First recorded measurement of an endogenous phasic dopamine transient in a freely moving, lesioned rat.
- First recorded measurement of a drug-evoked phasic dopamine transient in a freely moving, lesioned rat.
- First recorded measurement of voltammetry and electrophysiology at the same carbon-fiber microelectrode in an anesthetized, lesioned rat.
- First recorded measurement of voltammetry and electrophysiology at the same carbon-fiber microelectrode in a freely moving, non-lesioned lesioned rat.

Reportable Outcomes

Cheer JF, Heien MLAV, Garris PA, Carelli RM and Wightman RM. Simultaneous measurements of dopamine release and nucleus accumbens cell firing at the same probe reveal different temporal scales of signal encoding in awake rats. Manuscript submitted to Journal of Neuroscience. **Appendix V.**

Howes GA, Boeckmann LB, Greco PG, Pakdeeronachit S, Sandberg SG and Garris PA. 2005. Comparison of "traditional" and "extended" waveforms used with fast-scan cyclic voltammetry for modeling dopamine release and uptake in the rat striatum. Abstract submitted to the Annual Meeting for the Society for Neuroscience (Washington DC). **Appendix VII.**

Mithyantha J. 2005. Behavioral correlates of partial degeneration of nigrostriatal dopaminergic neurons in the rat. M.S. Thesis. **Appendix I.**

Mithyantha J, Pakdeeronachit S, Woodlee MT, Schallert T and Garris PA. Partial unilateral nigrostriatal dopamine denervation in the rat: behavioral correlates potentially associated with reduced phasic dopamine signaling. Manuscript in preparation.

Sandberg SG, Heidenreich BA and Garris PA. 2005. A methodology for *in vivo* detection of the effects of dopamine transients on glutamate-evoked striatal activity using electrophysiological, fast-scan cyclic voltammetric and micro-iontophoretic techniques.

Abstract submitted to the Annual Meeting for the Society for Neuroscience (Washington DC). **Appendix IV.**

Schallert T & Woodlee MT (2005). Orienting and placing. In Whishaw IQ & Kolb B. *The behavior of the laboratory rat*. New York: Oxford University Press, pp 129-140. **Appendix II.**

Venton BJ, Sandberg SG, Binoy ED, Bungay P, Bezard E, Wightman RM and Garris PA. "Passive Stabilization" of striatal dopaminergic tone across the preclinical denervation range of Parkinson's disease: a theoretical study. Manuscript in preparation. **Appendix VI.**

Woodlee MT, Kane JR and Schallert T. 2005. A behavioral marker of non-dopaminergic damage in the 6-OHDA model of Parkinson's disease. Abstract submitted to the Annual Meeting for the Society for Neuroscience (Washington DC). **Appendix III.**

Woodlee MT, Mithyantha J, Kane JR, Chang J, Garris PA and Schallert T. Functional reorganization of the intact hemisphere following unilateral nigrostriatal dopamine depletion: implications for Parkinsonian models. Manuscript in preparation.

Conclusions

Work directed at developing behavioral tests sensitive to the partial dopamine depletion mimicking the preclinical phase of Parkinson's disease is progressing well. This aspect of the project has been expanded from the original proposal. These tests will provide both insight into the function of phasic dopaminergic signaling on their own and the opportunity for simultaneous assessment of behavior and dopamine in years three and four.

The first recording of electrically evoked dopamine levels, spontaneous phasic dopamine transients, and drug-induced dopamine transients in a freely moving, lesioned animal has been established. These measurements are now becoming routine and will be used in years three and four to investigate whether dopamine transients are affected by partial dopamine denervation as hypothesized. This approach will also be used to monitor dopamine during behavioral tests sensitive to partial dopamine denervation as described above. Continued development of a higher throughput lesion procedure will advance this goal.

The first recording of voltammetry and electrophysiology at the same carbon-fiber microelectrode was collected in the anesthetized, lesioned rat and freely moving, intact rat. Preliminary data in the anesthetized rat suggests that lesions cause a switch from transient inhibition to excitation by dopamine in striatal units. Work on this aspect of the proposal will additionally focus on electrically evoked dopamine effects during the third and fourth year. The establishment of combined voltammetry and electrophysiology in the freely moving, intact rat is a technical milestone that will spur similar measurements in freely moving, lesioned rats in the final two years of the proposal. Taken together, these results indicate that the necessary technical development to achieve the proposed goals of the project has now been established.

Finally, not needing to focus on technical development during years three and four, we will be able to spend more time preparing and submitting manuscripts.

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CHAPTER I

REVIEW OF LITERATURE

Abstract

Classic motor symptoms of Parkinson's disease (PD) do not appear until patients have severe losses (greater than 80%) of striatal dopamine content. The period prior to this level of dopamine loss constitutes the pre-clinical stage of the disease. The appearance of obvious motor symptoms heralds the onset of the clinical stage of PD. During the pre-clinical stage patients have few symptoms, but there are reports that they may have mild disturbances of movement and some non-motor deficits such as changes in olfaction, mood and cognition. Similar to the appearance of symptoms during the pre-clinical stage of PD in humans, animal studies have shown the appearance of behavioral deficits in animals with partial lesions of the nigrostriatal dopaminergic system (less than 80% striatal dopamine loss). This literature review addresses the symptomatology of pre-clinical and early stages of PD and animal studies where behavioral deficits have appeared at relatively moderate degrees of dopamine denervation (in the 20-80% depletion range). In order to discuss behaviors associated with dopamine denervation, it is important to address the behavioral functions of the caudate-putamen. Therefore, the functional heterogeneity of the caudate-putamen in terms of the effects of various regional lesions on different behaviors is also discussed. Finally, also discussed are the modes of dopamine neurotransmission and their role in behavior. One mode of

dopaminergic signaling that is important for behavior - phasic signaling - appears to decrease proportionally with dopamine denervation. Our overall hypothesis is that pre-clinical behavioral deficits are related to a reduction phasic dopaminergic signaling. Studying the behaviors mediated by phasic signaling could contribute towards a better understanding of the pre-clinical phase of PD.

Introduction

Parkinson's disease (PD) is a debilitating neurodegenerative disorder affecting 1% of the population over the age of 55 years. It is associated with the loss of dopaminergic neurons in the nigrostriatal pathway. Cardinal symptoms of PD are resting tremor, rigidity, bradykinesia and postural instability [1]. Other prominent features include a bent posture, short-stepped gait with decreased associated arm swing, decreased facial expression ("mask-like face"), and changes in the quality of speech. Speech may become soft, unclear, with lack of intonation and frequent halting. Handwriting progressively deteriorates, becoming small and illegible (micrographia). Neurological examination demonstrates no change in muscle strength and tendon reflexes. Along with the above motor deficits, mental disturbances including cognitive deficits, dementia and depression may also be present in some patients [2].

The diagnostic symptoms of Parkinson's disease do not begin to appear until about 80% of striatal dopamine content is lost [3]. Age-dependent reductions in substantia nigra dopamine neurons occur at a rate of about 5% per decade [4]. It was believed previously that this age-dependent loss of neurons progresses at an accelerated rate in some people, causing them to lose enough neurons to reach a symptomatic threshold during their lifetime. However, after onset of symptoms, the rate of cell death

measured by histological methods is about 45% per decade. Also, measurement of striatal dopamine metabolism by positron emission tomography (PET) and single-photon emission computed tomography (SPECT) in patients with PD has shown that the rate of loss of dopamine function is even greater (about 11% per year) [5]. By extending this exponential loss of dopamine innervation back in time, it has been estimated that neuronal degeneration begins not more than 4-7 years before symptoms become apparent ([6], reviewed in [7]).

Although there is no cure, various treatments are available for PD. Most drug treatments are aimed at replacing dopaminergic transmission in the striatum. These include exogenous administration of levodopa, direct dopamine agonists, or inhibitors of dopamine breakdown such as selegiline (MAO inhibitor), and tolcapone and entacapone (COMT inhibitors) [1]. Drugs such as amantadine and anticholinergics, targeting non-dopaminergic systems, are also useful. However, chronic use of these drugs is associated with many debilitating side effects including dyskinesias (involuntary movements), psychotic symptoms and confusion. Disease progression is not halted by any drug treatment. Surgical approaches, including ablation or high-frequency stimulation of the subthalamic nucleus and thalamus may be necessary, but may not be successful in improving all the symptoms of the disease. Also, surgical treatments are expensive and associated with all the risks surrounding highly invasive surgery, especially infections and intracranial bleeding [8]. Transplantation of dopaminergic tissues such as autologous adrenal medullary tissue and fetal mesencephalic tissue into the putamen or caudate nucleus have been tried, but all patients may not benefit, and some symptoms may return

within a year or two of the surgery [9]. Therefore, all the treatments available are useful only to a certain extent, and do not offer complete and permanent recovery of function.

None of the pharmacological and surgical treatments described above have been shown to have any effect on disease progression. Consequently, neuroprotective treatment to prevent or slow down neuronal loss is under investigation. Among the compounds being tested for efficacy in neuroprotection are antioxidants, anti-inflammatory compounds, neurotrophic factors and anti-apoptotic compounds [10]. Although none of these putative neuroprotective drugs is recommended for preventing the disease in human subjects at this time, the availability of neuroprotective therapy may not be very far away.

Currently, a diagnosis of PD is made when the classical motor symptoms develop. At this stage, the neuropathology is far advanced and most of the striatal dopamine content has been already lost. Therefore, when patients are started on treatment, only a small percentage of nigrostriatal dopamine neurons are remaining, and these neurons are still undergoing further degeneration. Therefore, any drug that aims to replace dopamine by increasing function of these remaining neurons has only a small population to act on, and may not be effective after some time. If the diagnosis of PD is made before obvious symptoms develop, there is a larger population of dopaminergic neurons that have not yet been lost. Attempts at neuroprotection may be more effective during this pre-clinical stage. Therefore, establishing a diagnosis of PD at an early stage in the disease process is specially important.

Once PD has been diagnosed, no currently available treatment can prevent further degeneration of neurons. However, there is some indication that if PD is diagnosed early

enough, physical therapy or some form of forced physical activity may be able to prevent further neuronal degeneration. Such neuroprotective effects of forced limb use have been demonstrated in animals with unilateral PD induced by injection of 6-hydroxydopamine into the nigrostriatal dopaminergic pathway [11-13]. Animals that were forced to use the affected forelimb immediately after injection of the neurotoxin did not show behavioral and neurochemical changes associated with toxin-mediated damage to dopamine neurons. Also, neuroprotective therapies that prevent neuron loss may become available in the near future and patients with an early diagnosis of PD may benefit greatly from such treatment. Therefore, much effort has been made towards detecting PD at an earlier stage than current diagnostic methods allow.

Early signs and symptoms of PD

Although clinical diagnosis of PD is made based on the presence of classical motor symptoms, there have been attempts at detecting mild motor and non-motor symptoms that appear earlier than classical symptoms. Many studies have been directed towards making a definite diagnosis of PD as early as possible in patients who have some neurological motor symptoms that are not clearly parkinsonian. Detection of disease onset is made difficult by the gradual progression of symptoms which are often ignored by the patient and the caregivers. In most patients, the earliest symptoms of PD are often vague and are attributed to fatigue, stress, or mild depression [14]. In most cases, the disease remains undiagnosed until characteristic tremors appear. One method to detect patients in the early stages of the disease is by use of neurological tests that are more sensitive to subtle motor slowing. Tasks involving visuomotor control such as tracking and tracing a moving cursor with the hands are slowed patients with early PD [15]. Since

such activity requires greater skill and co-ordination than the simple motor abilities tested in a regular clinical examination, deficits may appear before obvious motor symptoms are noticed. In one study of hemiparkinsonian patients, both the affected and the unaffected hands were significantly slower and made more errors in tracking and tracing tasks than control subjects, although the classical motor symptoms were present only on one side of the body [16]. Therefore, performance of tasks requiring visual control of hand movement may be a valuable test to detect cases of early PD.

Non-motor symptoms including changes in olfaction, cognition and mood may appear earlier than motor symptoms in many patients. A decreased sense of smell is an early sign of PD [17;18]. Various reports have described deficits in different modes of olfaction, including odor detection, identification and discrimination. In one study, odor discrimination was found to be negatively correlated with the severity of disease [18]. Also, subtle cognitive deficits such as mental slowing and poor performance on neurpsychological tests may appear in the early stages of PD [14;19;20]. Early deficits in cognition have been studied in subjects who had exposure to MPTP. MPTP is a byproduct during the manufacture of synthetic heroin. In the brain, it gets converted MPP^+, a toxic metabolite which selectively destroys dopaminergic neurons and causes parkinsonian symptoms. Subjects with MPTP exposure, but without obvious motor signs of parkinsonism show deficits in cognitive ability comparable to that of exposed subjects who do show motor signs [21]. In this study, the subjects exposed to MPTP did have subtle parkinsonian symptoms, but did not meet the criteria for diagnosis of PD. Therefore, the cases described in this study may depict an early stage of clinical PD and not the pre-clinical phase. Depression is present in many patients with early PD [22]. A

biphasic distribution of depression has been reported in PD, with some patients having depression in the early stages, and in others when the disease is advanced. In patients with early PD, depression is thought to be related to the neurodegenerative process that is associated with the disease. However, in patients with severe symptoms, depression may be related to a decreased ability to perform the activities of daily living [23].

Since any single screening tool may not be very sensitive in detecting the early stages of PD, some authors have evaluated a series of clinical tests in combination as a screening tool to help detect patients in the early stages of the disease before the appearance of full blown motor symptoms. One such study combined tests to detect slowing of movement, decreased sense of smell and depression [24]. Motor function was assessed by a wrist flexion–extension task that required subjects to move their wrist to point to certain targets as rapidly and accurately as they could. Olfaction was assessed by the University of Pennsylvania Smell Identification Test (UPSIT) which requires identification of standardized odors. Screening for depression was carried out using the Beck Depression Inventory. These tests in combination reliably separated patients who were recently diagnosed with early-stage PD from normal controls. The same battery of tests was applied to subjects with some neurological symptoms but not meeting the diagnostic criteria for PD, and follow-up evaluations were conducted for at least 1 year [25]. About 30% of the subjects had clinical PD at the end of the evaluation period and of these, about 70% had had abnormal scores on the screening battery administered at least a year before the follow-up evaluation. Therefore, this set of tests increased the sensitivity of PD case detection.

"Pre-clinical" symptoms of PD in humans

The non-classical symptoms discussed above still fall within the realm of clinically detectable PD. However, some reports seem to indicate that certain subtle behavioral changes predate the earliest clinically detectable signs by a few years. These changes may be termed pre-clinical symptoms. For instance, in some patients, there were subtle deficits of movement, chronic exhaustion, episodes of transient muscular weakness, poor accuracy of movements and coordination, and a decrease in spontaneous mobility for many years preceding diagnosis [14]. A classic example is that of a patient who had a self-winding watch which stopped working a few days after she started wearing it on her left wrist [26]. It worked perfectly when others wore it, and when she wore it on her right wrist. Three years later, she developed tremors and difficulty in moving in her left arm and was diagnosed as having Parkinson's disease. In another case, a soccer player who was diagnosed with the disease had been observed to have intermittent subtle abnormalities of movement for many years before diagnosis [14]. Old video footage of his soccer games showed abnormal movements of his right arm and leg ten years before he had classical parkinsonian symptoms. He also noticed an involuntary tendency to swerve towards the right while driving and swimming. However, in both these cases, it is not known if the symptoms were truly "pre-clinical", i.e., there were no deficits on any neurological tests, or if the disease was not diagnosed early enough despite the presence of motor signs that could have been detected by careful testing if the patient had approached health care professionals earlier.

Subtle changes in motor performance may manifest as a change in speech and may appear before overt symptoms of PD [27]. Speech may become slow, soft or hoarse

many years before diagnosis. In one case study, old video clips (from six years prior to diagnosis) of a patient were obtained and compared with that of a control subject by computerized analysis of voice samples [28]. The fundamental frequency of the patient's voice had significantly lesser variability than that of the control for the five years prior to diagnosis and a year after diagnosis but before treatment was started. The variability in frequency during this period was also significantly reduced when compared to the baseline value in the previous year and in the two years after treatment was started. Again, it is not known if there were mild motor symptoms of PD before a clinical diagnosis was made, but the patient was not started on any treatment for two years after the diagnosis. Therefore, his case might have been mild when diagnosed, and the speech defects may have been truly pre-clinical.

Another motor function that may be affected early in PD is the ability to write [27]. Changes in handwriting, with the letters becoming smaller and illegible, may occur relatively early in the disease process, before other motor symptoms become apparent. Since most patients have some samples of handwriting over the years, it may be possible to analyze changes in handwriting by comparing present handwriting with previous examples in order to make an early diagnosis of PD.

Dementia may occur in some patients with PD [29;30]. Subtle cognitive deficits such as impairments in sequential tasks, visuospatial deficits and deficits in attention shifting and learning ability may be present in a larger number of patients[31-37]. In one study cognitive deficits were seen in asymptomatic twins of patients with the disease [38]. Another study showed abnormalities on neuropsychological testing in roughly 30% of asymptomatic first degree relatives of patients with familial PD [39]. These studies

indicate that cognitive slowing may be present during the pre-clinical stage. However, no prospective studies have been conducted to determine the occurrence of PD in those asymptomatic subjects who had poor scores on tests of cognition.

Disorders of mood including depression and anxiety are other non-classical symptoms that may be present before motor symptoms develop. In one study, about 44 % of patients reported episodes of depression before obvious PD symptoms appeared [22]. Anxiety disorders also occur in many patients, and may be present for many years before diagnosis of PD [40].

Assessing striatal dopamine innervation might resolve the issue of differentiating between pre-clinical and early PD. In pre-clinical cases of PD, brain imaging would show a reduction in striatal dopaminergic activity to below the levels typical in normal subjects, but greater than that of patients with early, mildly symptomatic clinical PD. One way to measure dopaminergic activity in live subjects is by positron emission tomography (PET) or single photon emission computed tomography (SPECT). These imaging techniques make use of radio-labeled compounds to measure the uptake of [^{18}F] fluorodopa into dopaminergic neurons, binding of [^{123}I] β CIT to dopamine transporters in the striatum, or quantify dopamine in synaptic vesicles by measuring activity of vesicular monoamine transporter 2 (VMAT2) using (+)-[^{11}C] dihydrotetrabenazine [41]. PET and SPECT studies have shown a loss of dopaminergic terminals in the striatum of patients even in the early stages of the disease [42;43] and in some asymptomatic twins and other first degree relatives of patients with the disease [7;44-46]. Although imaging studies may be useful in detecting PD at a preclinical stage, they are expensive to use as a screening tool for the general population and require expertise that may not be readily

available, and also involve exposure to radiation. But if used in conjunction with easily administered tests such as tests of olfaction, these imaging techniques could be very a useful tool in detecting pre-symptomatic PD cases.

Some investigators have used imaging techniques to look at olfactory function in conjunction with striatal dopaminergic activity in first degree relatives of patients with the disease. In one study, first degree relatives were screened for hyposmia by odor detection, discrimination and identification tests [47]. Hyposmic individuals were subject to SPECT scanning with [^{123}I] β CIT binding to quantify striatal dopamine transporters. Out of 25 hyposmic relatives, four showed a marked decrease in striatal [^{123}I] β CIT binding, while none of the normosmic relatives had any decrease in binding. Two of these four individuals later developed classical PD symptoms. A prospective study examined asymptomatic relatives of patients with PD by a combination of olfactory tests and SPECT using [^{123}I] β CIT binding, and followed them up two years later with SPECT, clinical evaluation and a questionnaire sensitive to the presence of PD [48]. Among the hyposmic relatives, 10% who had greatly reduced [^{123}I] β CIT binding at baseline developed clinical PD, while none of the others did. Even in the hyposmic relatives that did not develop the disease, the rate of binding was significantly lower than in the normosmic relatives.

Another method to detect disease in asymptomatic subjects is a series of tests all of which are sensitive to changes associated with early PD. One study which evaluated a combination of tests for motor function, olfaction and mood was applied to asymptomatic first degree relatives of patients with PD [49]. A significantly greater percentage of first degree relatives than controls had scores in the abnormal range. When individual subtests

were analyzed, scores on the tests for olfaction and depression were significantly different between first degree relatives and controls.

As described above, studies on asymptomatic twins and first degree relatives of patients with PD have shown that measures such as olfaction, cognition and mood may be affected even when motor symptoms of PD have not developed and that subjects with such deficits are at greater risk for development of classical PD at a later time. Such tests applied to the general population during routine physical examinations could help in detection of incipient PD, without the presence of any motor signs. There have been no prospective studies on asymptomatic subjects in a general population to assess the validity of these tests as effective screening methods. However, the recognition of these sub-clinical features in populations at higher risk for developing PD (such as asymptomatic first degree relatives and twins) but without any clinical features suggesting motor impairments points to the fact that there are deficits that appear in the absence of the diagnostic symptoms of PD.

Behavioral tests that detect severe dopamine depletion in animal models of PD

Most studies in animal models of PD have been performed on animals with severe depletion of dopamine in the striatum to mimic the massive loss of dopamine in human cases. Behavioral tests have been used to assess the severity of dopamine neuron loss in these animals. One of the most common behaviors tested is rotation induced by injection of apomorphine (reviewed in [50]). In rats with unilateral depletion of dopamine, there is an up-regulation of dopamine receptors on the lesioned side. Therefore, when a direct dopamine agonist such as apomorphine is injected, the animals have disproportionate increase in dopamine receptor activation on the lesioned side and show turning behavior

towards the side of the body being controlled by the lesioned striatum (contralateral to the lesioned side). This increase in contralateral rotation is seen only in animals with severe depletions of striatal dopamine (>80% depletion). At this level of depletion, obvious motor symptoms of PD appear in human cases. Another behavioral test used to detect severe lesions is the elevated body swing test [51;52]. Here, rats with unilateral striatal dopamine depletion are held up vertically by their tails and raised an inch above the cage floor. The direction of swinging movements of the rats' heads was recorded. Rats with severe depletions of striatal dopamine show a contralesional bias in the swinging movement. Animals with severe depletions of dopamine exhibit signs of akinesia [53]. This is tested by holding the rats such that only one forelimb was placed on the testing surface at a time and all the body weight is borne on that limb. This forelimb is allowed to step freely, and the number of steps taken by ipsilesional and contralesional forelimbs within a certain time period is counted. Akinetic animals have a difficulty in initiating weight-shifting movement in the affected forelimb, and have a greatly decreased step count on that forelimb as compared to the unaffected forelimb. Forelimb placing evoked by stimulation of vibrissae is another behavior that is lost in animals with severe depletions of striatal dopamine [54;55]. An additional behavioral deficit that is seen in severe lesions is an increased latency to disengage from eating to respond to a stimulus applied to the vibrissae [56;57]. These last two behavioral tests have been conducted mainly in animals with severe lesions, and the effect of partial dopamine denervation is not established.

Behavioral deficits associated with partial dopamine denervation in animal models of PD

Although most classic symptoms of Parkinson's disease appear when more than 80% of the striatal dopamine content is lost, there is emerging evidence demonstrating behavioral deficits in animals with a partial degree (<80%) of denervation of striatal dopamine. One study examined asymmetry of forelimb usage in rats with unilateral depletion of striatal dopamine during vertical exploratory behavior in a Plexiglas cylinder. The number of times each forepaw was used to contact the cylinder wall during rears was counted. The percent use of the forelimb unaffected by the lesion was greater than that of the affected forelimb and this asymmetry was shown to be significantly increased from baseline in rats with 30-70% loss of striatal dopamine content [53].

Reactive capacity is another behavioral measure that seems to be sensitive to partial dopaminergic denervation. Rats trained to hold a lever down and then release it quickly in order to avoid a mild footshock showed significant decrease in the percentage of successful escapes from footshock after only 11-24% dopamine depletion in the striatum [58]. Tasks of fixed ratio bar pressing for food rewards also identified deficits (reduced bar presses) in rats with moderate striatal dopamine denervation [59]. In this study, the striatal lesion was partial (total striatal dopamine content 33% of control) and graded, with the ventrolateral striatum being more lesioned than the dorsomedial part (mean dopamine content 18% and 49% of control in the ventrolateral and dorsomedial striatum, respectively). This study also showed deficits in forelimb placing and in a test designed to assess forelimb rigidity and akinesia on passive lateral movement in animals that were moderately lesioned. In another study, percentage of correct responses on a bar-

pressing task to obtain food reward decreased with increasing degrees of dopamine denervation, beginning at about 40% depletion [60].

Studies on primates have also shown the appearance of behavioral deficits at moderate degrees of dopamine depletion. In one study, monkeys with unilateral 6-OHDA lesions demonstrated asymmetry in limb use and the position of the head at rest [61]. A linear relationship was seen between ipsilateral bias in head position and the density of tyrosine hydroxylase immunoreactivity in the putamen, in a range of partial dopamine denervation (40-80% depletion). In studies involving monkeys that received MPTP injections over a period of time to simulate the progression of PD, cognitive deficits such as a difficulty in predicting the best path around an obstacle in reaching tasks and persistence in performance of tasks that were not rewarded appeared earlier than motor symptoms of PD [62;63]. However, the degree of dopamine denervation at which the cognitive deficits appeared is not known. Another study showed similar results, with changes in saccadic eye movements and deficits in reversal of a previously rewarded task appearing when smaller doses of MPTP were administered such that obvious parkinsonian symptoms did not develop [64]. Tyrosine hydroxylase immunoreactivity showed that 70-80% of dopaminergic neurons in the substantia nigra were lost in these animals. However, in human cases, it is thought that classical parkinsonian symptoms appear with about 50% loss of dopaminergic neurons in the substantia nigra [4]. Further, another single injection of MPTP in one of the monkeys in the above study brought about the occurrence of tremor, rigidity and akinesia. This means that the primates showing cognitive deficits in this study did have a severe degree of neuron loss in the substantia nigra, although severe motor symptoms of PD may not have appeared.

Functional heterogeneity of the caudate-putamen

In order to discuss the behavioral effects of caudate-putamen (CP) dopamine depletion, it is important to first identify the main functions of the CP with respect to behavior. The CP of rats is a single anatomical structure comprising the caudate nucleus and putamen, two separate nuclei in the primate and human brain. The medial CP corresponds to the caudate nucleus and the lateral CP corresponds to the putamen. The afferent innervation of different sub-regions of the striatum is heterogenous [65]. The dorsolateral parts of the CP have afferent inputs from the sensorimotor cortex and from the dopaminergic neurons of the substantia nigra. The ventromedial parts receive cortical afferents from the anterior cingulate cortex and pre-frontal cortex, and dopaminergic input from the ventral tegmental area. This difference in innervation within the CP may lead to differential behavioral effects of dopamine depletion affecting these sub-regions.

Some investigators have studied the behavioral deficits that appear in rats with site-specific lesions of the striatum, obtained by local injections of neurotoxins into various striatal sub-regions. The neurotoxins used include 6-hydroxydopamine (6-OHDA), ibotenic acid, and kainic acid. 6-OHDA injection into the striatum causes degeneration of dopaminergic nerve terminals in the striatum and of the cell bodies in the substantia nigra [66;67]. Ibotenic and kainic acid injections destroy cell bodies in the striatum, but leave intact the axons that pass through the region [68;69]. However, lesions caused by all these neurotoxins alter sub-regional functions of the CP, and have similar behavioral effects in most tests. In most of the lesion studies described below, the site-specific loss of neurons was severe (>70-80% loss) unless otherwise indicated.

Lateral and ventro-lateral lesions of the caudate-putamen

Feeding behavior is often impaired in animals with lesions of the striatum. Severe aphagia and adipsia were noted in rats with kainic acid lesions of the posterolateral striatum [68]. Rats with bilateral 6-OHDA lesions of the ventrolateral striatum (VLS) showed decreased food and water intake during the first 3-4 days after lesion surgery compared to animals with lesions of the dorsolateral striatum (DLS) and the anterior ventromedial striatum (AVMS) [70-73]. Similar deficits were seen in rats with ibotenic acid lesions of the rostrolateral striatum [74]. Food intake was significantly correlated to the dopamine content in the VLS in rats with 30-75% lesions of the VLS [70] or to that in the lateral striatum in rats with severe losses of neurons in this region (dopamine content of lesioned side <10% of the intact side) [75].

Ibotenic acid and 6-OHDA lesions of the lateral striatum or the VLS, but not the medial striatum, cause deficits in forepaw use that manifest as difficulty in holding and manipulating food pellets, and performance of skilled forepaw reaching tasks [69;74;76-78]. Forelimb use for stepping was seen to be reduced in animals with 6-OHDA lesions of the lateral and ventral parts of the striatum but not of the dorsomedial parts [79]. Transplants of embryonic substantia nigra grafts that innervate the ventrolateral striatum in 6-OHDA lesioned animals improved performance on certain tests evaluating the use of limbs for placing, withdrawal and clasping abilities, whereas grafts innervating the dorsal striatum did not [80].

Lesions of the ventrolateral and lateral striatum also seem to impair movement of orofacial structures. VLS lesions caused by striatal injection of 6-OHDA produce cheek tremor and "vacuous chewing", a type of movement where the lower jaw moves

repeatedly in a vertical motion, similar to chewing [70]. Deficits of tongue protrusion and licking activity appear in ibotenate lesions of the striatum, but are persistent only in lesions of the lateral striatum and not those of the medial striatum [77]. Also, rats with 6-OHDA induced VLS lesions were noticed to take smaller bites of food, scraping the pellets with their teeth [71] and rats with ibotenic acid lesions of the lateral striatum exhibited deficits in the use of teeth to pick up and to bite off food [74]. In this last study, dorsolateral striatal lesions caused deficits in forepaw use for holding food pellets, whereas ventrolateral lesions showed greater deficits in biting and consumption of food. In a similar study, but with less extensive ibotenate lesions of the striatal sub-regions, dorsolateral lesions caused forelimb reaching deficits, but had only transient effects on tongue protrusion and licking [81]. Ventrolateral lesions affected both forelimb reaching and tongue protrusion, but the forelimbs reaching deficits were less than that for dorsolateral lesions. Lesions of the dorsomedial striatum had no effect on either of these behaviors. Similarly, in marmosets with unilateral 6-OHDA lesions of the nigrostriatal tract, grafts of embryonic nigral tissue into the putamen reduced deficits in an arm reaching task, but grafts into the caudate nucleus did not [82]. Injection of amphetamine into the ventrolateral striatum caused appearance of orofacial stereotypy, but not injection into the medial parts of the striatum [83].These studies seem to indicate that the lateral striatum, particularly the ventral half, is important for control of movements of the forelimbs and the orofacial region.

Deficits in lever pressing tasks appear with regional lesions of the striatum. Decreased lever pressing on a schedule of fixed interval lever pressing for food reward was noticed in rats with 6-OHDA lesions of the VLS, but not with similar lesions of the

nucleus accumbens or medial striatum [84]. Maximal deficits in lever pressing in a fixed ratio schedule were observed in VLS lesions as compared to nucleus accumbens and medial striatal lesions [85]. The dopamine depletion in the VLS was severe (about 90% lesioned) and this dopamine content was correlated to the number of lever pressing responses in the first week after lesion surgery. Response initiation time was also increased in 6-OHDA induced lesions of the VLS [86;87]. In a conditioned reaction time task requiring release of the lever within a certain time interval after the stimulus to obtain food reward, rats with severe deficits were found to have denervation of a large portion of the striatum brought about by injection of 6-OHDA, whereas mild deficits were seen in rats with selective lesions involving only the dorsal striatum [88]. In one study, electrical activity was measured in the caudate-putamen during a conditioned avoidance response task. Some of the neurons recorded were activated by the stimulus, some were activated during the response and some were activated by both stimulus and response. It was found that most of the stimulus-related neurons were present in the medial striatum, and that the response-related neurons were more abundant in the lateral striatum [89]. It therefore appears that the ventral and lateral parts of the striatum are important for responding using the forepaw in lever-pressing tasks.

Animals with unilateral depletions of striatal dopamine often have increased latency in orienting towards a stimulus applied to the contralateral side of the body. This "sensorimotor neglect" has been thought to be not due to an inability to perceive the stimulus, but rather to a difficulty in initiating a motor response to it [90]. In rats with localized lesions of different parts of the striatum by injection of 6-OHDA or kainic acid, orientation to various stimuli applied contralateral to the side of lesion was reduced when

the midventral caudate-putamen (CP) was involved in the lesion. Lesions involving other parts of the CP did not produce significant reductions in the orienting response [91]. Injections of 6-OHDA into the lateral striatum caused deficits in sensorimotor orientation, but this deficit did not occur when the injections were in the medial striatum [92]. The authors also mention that the lateral CP in this study may include the midventral CP mentioned in the previous study (actually the ventrolateral CP at the anterior end of the globus pallidus). The sensorimotor orientation deficit in rats with almost complete dopamine denervation of the striatum was reversed by grafts of fetal ventral mesencephalon tissue placed in the ventrolateral part of the striatum, but not in the central striatum [93]. The deficit was also improved by grafts of embryonic SN tissue into the cortex so as to innervate the VLS, but not when the dorsal striatum was reinnervated [80]. Therefore, sensorimotor function seems to be dependent on the integrity of the ventrolateral part of the striatum.

Medial and dorso-medial lesions of the caudate-putamen

The spectrum of deficits produced by medial and dorso-medial lesions of the CP is different from that produced by lateral and ventro-lateral lesions. Animals trained in a T-maze task to obtain food reward on reaching the goal box were tested after kainic acid lesions of the anteromedial and ventrolateral caudate [94]. Ventrolateral lesions showed a decrease in the degree of spontaneous bias while anteromedial lesions showed no change in the degree of bias; 5 out of 8 animals with anteromedial lesions changed the direction of their bias and chose the side opposite to that chosen before lesion. Also, rats with anteromedial lesions made more errors than controls or rats with ventrolateral lesions in learning to reverse the bias towards the preferred side over five successive reversals.

Kainic acid lesions in the anteromedial striatum caused deficits in retention of a learned spatial task (delayed alternation) in a T-maze, while visual discrimination was unaffected [68]. Rats with ibotenic acid lesions of the medial striatum, but not of the lateral striatum, made significantly greater errors while learning to reverse a learned turn preference in a cross maze while having no deficits in acquisition or retention of the same task [69]. These rats had no deficits in learning to reverse a brightness preference and no deficits in forepaw reaching. Electrolytic lesions of the dorsal caudate nucleus impaired reversal of learning on a brightness discrimination task, and caused an increase in perseverative responses [95]. Therefore the integrity of medial and dorso-medial CP seems to be important for correct performance of various spatial tasks.

Another test that measures spatial memory is the Morris water task [96]. It is often used to test for different aspects of memory in rats. It consists of a swimming pool with a hidden or visible platform at one end, which the rats have to reach to escape from the water. The "place" task is carried out with the platform submerged in water, and the animal has to reach it without the use of visual cues, while in the "cue" task the rats can be guided by the platform visible above the water. Hippocampal lesions affect the "place" task but not the "cue" task. In a study which used the Morris water task on rats with bilateral ibotenic acid lesions of the medial striatum, lesions caused increased latencies in acquisition and retention of both place and cue tasks, and lesioned rats were found to use a different navigation strategy from controls [97;98].

In addition to deficits in spatial tasks, lesions in the medial CP affect other behaviors including general locomotion, rearing, rotational behavior and response to physiological stressors. Rats with near-complete 6-OHDA lesions of the anteromedial

caudate-putamen showed reduced response to a "physiological regulatory challenge", having reduced water intake after sub-cutaneous injection of hypertonic saline than animals with ventrolateral caudate lesions and control animals [73]. Animals with medial striatal 6-OHDA lesions also showed a reduction in general locomotor activity [72] and animals with dorsolateral striatal lesions showed a reduction in spontaneous rearing activity [70]. Another indication that the medial striatum is important for general locomotor activity is that in intact rats, injection of amphetamine into the medial striatum caused increased general locomotor activity and rearing while injections into the lateral striatum did not [83].

Rotational asymmetries induced by injection of drugs (amphetamine and apomorphine) and spontaneous rotational asymmetries may also be related to the depletion of dopamine in the medial and dorsal striatum. This has been studied in animals with grafts of fetal SN tissue to re-innervate the dorsal striatum. Such grafts improved spontaneous and drug- (amphetamine and apomorphine) and tail-pinch-induced rotational asymmetry whereas reinnervation of ventrolateral striatum did not [99]. In another study, grafts of fetal ventral mesencephalic tissue into the central part of the caudate-putamen reduced amphetamine-induced rotational asymmetry [93]. In a primate study, amphetamine-induced rotational asymmetry was improved by grafts of embryonic SN tissue into the caudate nucleus which probably corresponds to the dorsomedial CP, but not the putamen (probably corresponds to the ventrolateral CP) [82]. All these studies only demonstrate improvement in rotational asymmetries after grafting dopaminergic tissue into the medial and dorsal parts of the CP, but do not quantify asymmetries produced by isolated lesions of these sub-regions. A study in which isolated sub-regional

striatal lesions were tested contradicts these findings. In this study, amphetamine-induced rotational asymmetry was noted in animals with 6-OHDA lesions of the dorsolateral, ventrolateral and ventrocentral CP, but not with lesions of the dorsomedial, dorsocentral or ventromedial CP [79]. Therefore, the role of the medial and dorso-medial CP in the appearance of rotational asymmetries in unilaterally lesioned animals remains unresolved.

It appears therefore that the CP is functionally heterogenous within various sub-regions mediating different behaviors. To summarize, the medial CP seems important for learning and performance of spatial tasks, general activity levels and possibly rotational behavior, while the lateral CP seems to be important for control of movement of limbs and eating behavior. Therefore, the behavioral deficits that appear on lesioning the striatum depend on the striatal sub-region that is maximally affected.

Dopamine Neurotransmission

There are two modes of dopaminergic signaling in the striatum: tonic and phasic signaling [100-102]. Tonic signaling involves random firing of neurons, bringing about a constant steady-state level of extracellular dopamine, called the dopaminergic tone. Dopaminergic tone acts as a gate; a certain level of dopamine in the brain extracellular fluid is required to allow movement to occur. With increasing dopamine depletion, tone is maintained until about 80% of the striatal dopamine content has been lost [103-107]. Beyond this level of depletion, dopaminergic tone drops, and this reduction in tone is associated with the appearance of the classical motor symptoms of PD. Levodopa, a biochemical precursor of dopamine used in the treatment of PD, increases extracellular levels of dopamine (which is indicative of dopaminergic tone) measured in striatal slices

and in vivo by microdialysis [106;108;109]. In accordance with its role in increasing dopaminergic tone, levodopa brings about an improvement in behavioral deficits such as apomorphine-induced rotational asymmetry and akinesia caused by severe nigrostriatal dopamine depletion in animal models of PD [50;110].

Synchronized burst firing of neurons causes dopamine levels to spike on top of this dopaminergic tone (dopamine transients) and is called phasic signaling. Phasic signaling is said to be important for various aspects of behavior. Transient concentration spikes of dopamine have been recorded in rats when they were introduced into a novel environment [111], during sexual behavior [112], and during introduction of conspecifics [113]. Also, phasic signaling is said to mediate coding of error in reward prediction [101]. Another view holds that dopamine transients are important for switching attention and behavior towards salient stimuli [114]. Some recent evidence has shown that unlike dopaminergic tone, phasic signaling may not be maintained with dopamine denervation [115]. The amplitude of electrically evoked concentration spikes of dopamine (which may mimic spontaneous phasic firing) decreased proportionally with dopamine loss in the striatum. Therefore, there is some evidence showing that phasic signaling is reduced in proportion to dopamine neuron loss in the striatum.

Conclusion

From human and animal studies it appears that deficits in certain behaviors can appear much before obvious symptoms of PD develop. These deficits could potentially be associated with phasic dopamine signaling. Identification of behaviors putatively mediated by phasic signaling would help in a better understanding of the neural mechanisms underlying the symptomatology of PD.

CHAPTER II

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder associated with the loss of dopaminergic neurons in the nigrostriatal tract. The cardinal symptoms are resting tremor, rigidity, bradykinesia and postural instability [1]. These classical symptoms of PD do not appear until about 80% of striatal dopamine content is lost [3], and the appearance of these symptoms heralds the onset of the clinical stage of the disease. The stage of PD when the dopamine denervation is below the symptomatic threshold for classical symptoms is called the pre-clinical stage. However, mild motor and non-motor symptoms may appear in the pre-clinical stage, even years before the classical motor symptoms. Such symptoms include subtle intermittent disturbances in movement, reduction in olfactory function, abnormalities on neuropsychological testing suggestive of mild cognitive deficits, and mood disturbances [14;16;18-20;22;26]. Similar deficits have been detected in asymptomatic twins and other first degree relatives of patients with PD, suggesting that those subjects may be at increased risk for development of PD at a later time [38;39;47-49].

As with humans, lesion studies on animals have shown that for most of the sensorimotor behavioral tests used, obvious deficits appear when more than 80% of the striatal dopamine content has been lost. However some behaviors seem more sensitive to nigrostriatal dopamine denervation, with deficits appearing at less severe degrees of

lesion (i.e., equivalent to the pre-clinical range of denervation). One such test is the forelimb use asymmetry test or cylinder test which measures asymmetry in forelimb use during rearing movements in rats [53]. In this test, asymmetry has been shown to be significant for rats with 30-70% decreases of striatal dopamine content [53]. Another behavior that is sensitive to partial striatal dopamine denervation is reaction time in lever-pressing tasks [58-60]. Similar deficits appear in primates at pre-clinical ranges of dopamine denervation. For example, bias in head position has been shown to appear in the 40-80% striatal dopamine denervation range in hemiparkinsonian monkeys [61]. Also, in monkeys that received repeated injections of MPTP to simulate progression of PD, cognitive deficits, such as a difficulty in predicting the best path around an obstacle in reaching tasks, and persistence in performance of tasks that were not rewarded appeared before obvious motor symptoms of PD, for example, decreased general activity, tremor and rigidity [62;63].

It therefore appears that there are behavioral deficits that appear in both humans and animals at a lesser degree of dopamine denervation than required for classical parkinsonian symptoms to develop. One ramification of potential deficits during the pre-clinical stage is the possibility of early diagnosis of PD if the symptoms that occur in the pre-clinical stage can be evaluated. As mentioned earlier, patients have lost a major portion of their striatal dopamine innervation when obvious motor symptoms develop. However, if the disease were detected when the loss of neurons is less severe, treatments to prevent further degeneration could be instituted and might be more effective than if they were instituted later when most neurons have been lost.

There are two types of dopaminergic neurotransmission, tonic and phasic signaling [100-102]. Tonic signaling is brought about by low frequency unsynchronized firing of individual neurons, which produces a steady-state level of extracellular dopamine (dopaminergic tone) in the brain. A certain ambient extracellular level of dopamine is essential for movement to occur [116]. Phasic signaling involves synchronized high frequency firing of multiple dopamine neurons producing transient dopamine concentration spikes (phasic signaling or dopamine transients) above the tonic extracellular levels. This aspect of dopamine neurotransmission is important for various aspects of behavior, including the encoding of errors in reward prediction, switching between salient environmental stimuli, sexual behavior, and responding to novel stimuli and the introduction of conspecifics [101;111-114]. Tonic and phasic dopaminergic signaling differ in their response to dopamine denervation. Dopaminergic tone can be measured using the technique of microdialysis. A measurement of the ambient level of dopamine by microdialysis in animal studies shows that extracellular dopamine levels are normal until almost complete loss of dopaminergic terminals in the striatum, when these levels drop [103-107]. The reduction in dopamine tone at a severe degree of neuronal loss correlates with the appearance of classical parkinsonian symptoms in human patients and behavioral deficits in experimental animals at severe degrees of dopamine denervation. While the relationship of tonic signaling to denervation is well established, the effect of dopamine denervation on phasic signaling is less clear. Electrophysiological recording of substantia nigra dopamine neurons show that there is no reduction in burst firing of neurons until >96% of the striatal dopamine content has been lost [117]. However, the amplitude of electrically evoked dopamine concentration spikes, which may mimic

phasic signaling, decreases with increasing degrees of dopamine denervation [115]. Therefore, it is possible that behaviors mediated by phasic dopaminergic signaling may not be maintained at mild and moderate degrees of dopamine denervation in the striatum.

In both human subjects and animal models of the disease, it has been shown that behavioral changes can be detected at moderate degrees of neuronal loss, even though classical parkinsonian symptoms appear only after a severe degree of denervation. Our hypothesis is that these pre-clinical behavioral deficits are related to a reduction in phasic dopaminergic signaling which in turn is proportional to the loss of dopaminergic neurons. Therefore, behaviors that are linearly related to striatal dopamine content could be associated with phasic signaling and a deficit in these behaviors may mirror a decrease in phasic signaling.

In this study, a series of behavioral tests were evaluated in animals across the pre-clinical and clinical range of nigrostriatal dopamine denervation for PD to assess whether deficits were proportional to the degree of lesion. The tests were the forelimb use asymmetry test (cylinder test) [53;54], vibrissae-evoked placing [53;54], engagement and disengagement tests [56;57], and the passive initiation threshold test [118]. While most of the behavioral tests evaluated in this study have been previously performed on animals with severe lesions of the nigrostriatal dopaminergic projection, little information is available about these tests in partial lesions. The lesion model used in this study is a unilateral graded lesion of the substantia nigra (SN), which results in a graded loss of dopamine within the caudate-putamen (CP), with the lateral part most severely lesioned and the medial part least affected [52;115]. This difference of denervation within the CP is important because the innervation of various sub-regions of the CP is anatomically

heterogenous: the dorsolateral parts receive afferents from the sensorimotor cortex and the SN, and the ventromedial parts receive afferents from more medial and anterior parts of the cerebral cortex such as the prefrontal cortex and cingulate cortex, and the ventral tegmental area [65]. Due to this difference in innervation, the sub-regions may differ in their functional role in behavior. A graded lesion may therefore allow us to obtain different degrees of denervation of the CP sub-regions in the same animal; correlation of dopamine content of CP sub-regions to the behavioral deficits would help determine the sub-region whose dopamine content most accurately reflects the behavioral deficit. Also, it was necessary to compare our graded lesion with the more standard uniform MFB lesion in terms of the behavioral effects produced.

Among the behavioral tests assessed here, the limb use asymmetry test or cylinder test has been evaluated previously in animals with partial lesions of the nigrostriatal dopaminergic projection with injections of 6-OHDA into the medial forebrain bundle or the striatum [53], but not in partial graded lesions of the substantia nigra. The passive initiation threshold (PIT) test is a newly developed test of sensorimotor function and the effect of lesions on this test has not been previously estimated. Therefore, the main goals of this study were (1) to evaluate the effect of partial graded 6-hyrdoxydopamine (6-OHDA) SN lesions on these behavioral tests, (2) to determine the most appropriate sub-region CP for association with behavioral deficits and (3) to compare these lesions to the more commonly used severe lesions obtained by injection of 6-OHDA into the medial forebrain bundle (MFB).

CHAPTER III

MATERIALS AND METHODS

Animals

Adult male rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 250-400 g were used for the study. The animals were housed under standard conditions of lighting, temperature and humidity, and given food and water *ad libitum*.

General experimental design

All rats were handled and familiarized with the behavioral tests for two weeks before the lesion surgery was performed. The cylinder test was conducted on naïve rats to determine their preferred forelimb, and therefore the dominant hemisphere. Lesioning the dominant side of the brain rather than the non-dominant side causes a greater range of deficits when compared to pre-lesion performance on the behavioral tests. Rats received 6-hydroxydopamine (6-OHDA) injections into the lateral part of the substantia nigra (SN) or into the medial forebrain bundle (MFB) of the dominant hemisphere so that maximal behavioral deficits over pre-lesion performance would be obtained. Post-lesion behavioral tests were conducted at least 2 weeks after lesion surgery when the lesion is maximal and stable [119]. After completion of behavioral tests, the CP was analyzed for dopamine content. Since lesions of the MFB can produce severe depletions of nucleus accumbens (NAc) dopamine, we additionally determined the dopamine content of the NAc for the rats with MFB lesions.

Lesion surgery

Two types of lesions were made. A total of 29 rats received graded lesions by injection of 6-OHDA into the lateral part of the SN, and 12 received severe lesions by injection of the neurotoxin into the MFB. Lesion surgery was performed as described previously [52]. Briefly, rats were anesthetized with Equithesin (6 ml/kg i.p.), immobilized in a stereotaxic apparatus (David Kopf Instruments, Tajunga, CA), and administered desipramine (25 mg/kg i.p.) and pargyline (40 mg/kg i.p.) 20 minutes prior to surgery. The required quantity of 6-OHDA was dissolved in 2μl of ascorbic acid in sterile saline solution, and injected over 10 minutes. For the SN lesions, the stereotaxic co-ordinates for the injection were -5.4 mm AP, $\pm$ 3 mm ML and -8.2 mm DV with bregma as the reference point. Rats received injections of 7, 14, or 17 μg of 6-OHDA. For the MFB lesions, three rats received 17μg 6-OHDA injections at -3.3mm AP, $\pm$ 1.7mm ML and -9mm DV from bregma, and the rest received 10 μg 6-OHDA injections at -4.6mm AP, $\pm$ 1.3mm ML and -8.2mm DV from bregma.

Behavioral tests

Cylinder test

Rats were placed in a Plexiglas cylinder (12 inches tall and 8 inches in diameter) and their movements videotaped for about 5 minutes or until they made about 25 movements along the walls [53;54]. Videotaping was done with a camera under a Plexiglas platform on which the cylinder was placed. Scoring was done later using slow motion and frame-by-frame functions. The behaviors scored were (1) individual use of the right or left forelimb to touch the wall of the cylinder during a rear and (b) concurrent

or near-concurrent use of both forelimbs to touch the cylinder wall during a rear and for sideways movements along the wall.

The percent use of the non-impaired forelimb, percent use of the impaired forelimb and percent use of both limbs with respect to the total number of limb usage scored were calculated using the following formula:

Ipsilateral % score= (Ipsi+ ½ both)/ (ipsi+contra+both) (1)

Contralateral % score= (contra+1/2 both)/ (ipsi+contra+both) (2)

A single limb use asymmetry score was obtained by subtracting the contralateral forelimb score from the ipsilateral forelimb score.

Vibrissae-evoked placing test

The rats were held by their torsos with their limbs allowed unrestricted movement, and gently moved up and down in space so that their muscles were relaxed and they offered no resistance to being held. Each forelimb was tested by brushing the ipsilateral vibrissae against the edge of a table top to elicit placing of the forelimb onto the edge of the table (direct placing). The forelimb not being tested at the time was gently drawn back and held during testing. The contralateral vibrissae were touched with a finger during placing to provide a light stimulus to worsen placing asymmetries [53;54]. A variant of this test was performed, where placing of the limb on stimulation of the contralateral vibrissae was tested (across-midline placing). In another variant, the head-on placing test, forelimb placing was elicited by stimulation of the midline ventral surface of the rat's head. The test was done in three sessions on three separate days. The number of successful placing reactions for each forelimb was recorded. The percent of unsuccessful placing responses was calculated for both impaired and non-impaired forelimbs.

Engagement and disengagement tests

Engagement and disengagement tests were carried out in wire mesh cages which allow the experimenter access to the rat regardless of its location in the cage [56;57]. The rats were regularly handled and familiarized to introduction of cotton-tipped wooden swabs into their home cage and when they were placed in the wire mesh cage. They were periodically fed a cheese snack in the wire mesh cage until they readily ate this food. For the performance of the test, a single rat was allowed to habituate in the wire mesh cage overnight, such that it considered the cage its home cage, and showed no exploratory behavior. During the engagement test, a cotton-tipped wooden swab was touched to vibrissae on each side of the animal at 1 second intervals until the animal oriented to the swab or 15 seconds elapsed, whichever came first. The test was videotaped and the latency to respond to the stimulus was recorded. In the disengagement test, the animal was given a piece of the snack to encourage eating. While it was eating, vibrissae stimulation was carried out as described above. Again, the latency to respond to the stimulus was measured. These tests were videotaped, and latency was measured using a stopwatch, while running the tape in slow motion at a third of real-time speed. For animals that responded in the disengagement test, testing was performed multiple times on each side to determine the number of trials to habituation. Habituation was deemed to have occurred when the animals did not respond to stimulation of vibrissae for 15 seconds. This number was recorded both before and after lesioning the animals for a total of 6 animals with SN lesions and 11 with MFB lesions.

Passive initiation threshold test (PIT test)

The rats were held by their torso with one forepaw placed on the table top such that all their weight was borne on that forelimb [118]. They were then slowly moved forwards until the forelimb bearing weight stepped forward ("forward PIT"). The distance traversed by the tip of the snout before a forward step was placed was measured. The same test was repeated with the rats being moved towards either side (same and opposite side as the forelimb bearing weight- "same side PIT" and "opposite side PIT"). Testing was conducted on a rough paper surface to prevent the rats from dragging the forelimb being tested. Lines marking distance were drawn on this paper for ease of measurement of scores. The test was videotaped for accurate scoring. Two such sessions were performed both before and after lesion surgery.

Tissue Dopamine Content

Tissue dopamine content was determined as previously described [52]. Briefly, the rats were deeply anesthetized with urethane (1.5 g /kg i.p.) and decapitated. The brain was removed, chilled, placed in a brain block, and sliced into 1 mm sections. The slices that contain the CP were isolated, and dissected to obtain three $1mm^3$ sections (medial, medio-lateral, and lateral) from the dorsal half of the caudate-putamen (CP). For the MFB lesions, samples were also taken from the NAc. All samples were homogenized in a solution of dihydroxy benzyl amine (DHBA; – used as an internal standard) dissolved in 0.1 N perchloric acid (PCA), frozen on dry ice, then thawed and centrifuged. The protein content of the precipitate was determined using a Bio-Rad kit (Hercules, CA). The supernatant was used to determine dopamine levels by HPLC with electrochemical detection (BAS 200B with a Unijet detector and injector, Bioanalytical Systems, W.

Lafayette, IN) using a microbore, reverse phase column. Mobile phase consisted of 0.5g EDTA, 0.4g octane sulfonic acid, 24.56g of monochloroacetic acid, 1.16g of sodium chloride and 50 ml of acetonitrile in 2 L of water, at a pH of 4.0 and was pumped at the rate of 1 ml/min. The dopamine content of each section was expressed as mg dopamine/ mg protein. The dopamine content of a section from the lesioned side was divided by that of the corresponding section on the intact side to obtain the percentage dopamine innervation of the lesioned side.

Data analysis

Animals were grouped based on their average CP dopamine content into mild, moderate and severe lesions. Since symptoms of PD in humans appear when over 80% of striatal dopamine content is lost, animals with 80% or greater loss of striatal dopamine would show deficits related to the classical symptoms of PD, and were therefore deemed to have severe lesions (i.e., CP dopamine content in the striatum was <20% of the intact side). On the forelimb use asymmetry test it was observed that animals with less than 20% lesions did not have any deficits and were comparable to non-lesioned animals (T. Schallert, personal communication). Therefore, in our study such animals were said to have mild lesions (CP dopamine content >80% of the intact side). Our main aim was to study the behavioral correlates of partial lesions, i.e., those lesions that show deficits but do not show severe parkinsonian symptoms such as akinesia and rigidity. Animals whose CP dopamine content of the lesioned side was 20-80% of the intact side would fall under this category of pre-clinical denervation range and were termed the moderate lesion group. The mean scores on the behavioral tests for each group were compared by ANOVA and post-hoc t-tests with a Bonferroni correction. Mean dopamine contents of

the medial, medio-lateral and lateral dorsal CP were also compared by ANOVA. For the cylinder test, simple linear regression was applied and the correlation coefficient calculated. All the other tests showed a clustering of the animals into two main groups: those that showed significant deficits and those that did not. Since deficits did not appear to be linearly related to CP dopamine content for these tests, regression analysis was not done, but animals were grouped into categories based on CP sub-region dopamine content as mentioned above. Two-way ANOVA was used to compare differences in CP sub-region dopamine content and responses on certain tests (vibrissae-evoked placing and disengagement tests). Of the 12 animals that received MFB injections, one was not lesioned. Since our aim was to compare the differences between graded lesions obtained by SN injection and severe dopamine depletions obtained by MFB injection, we did not include this animal in the data analysis. All statistics were done using SAS (Cary, N.C.) and Microsoft EXCEL software. For all comparisons, the significance level (p) was set at 0.05.

CHAPTER IV

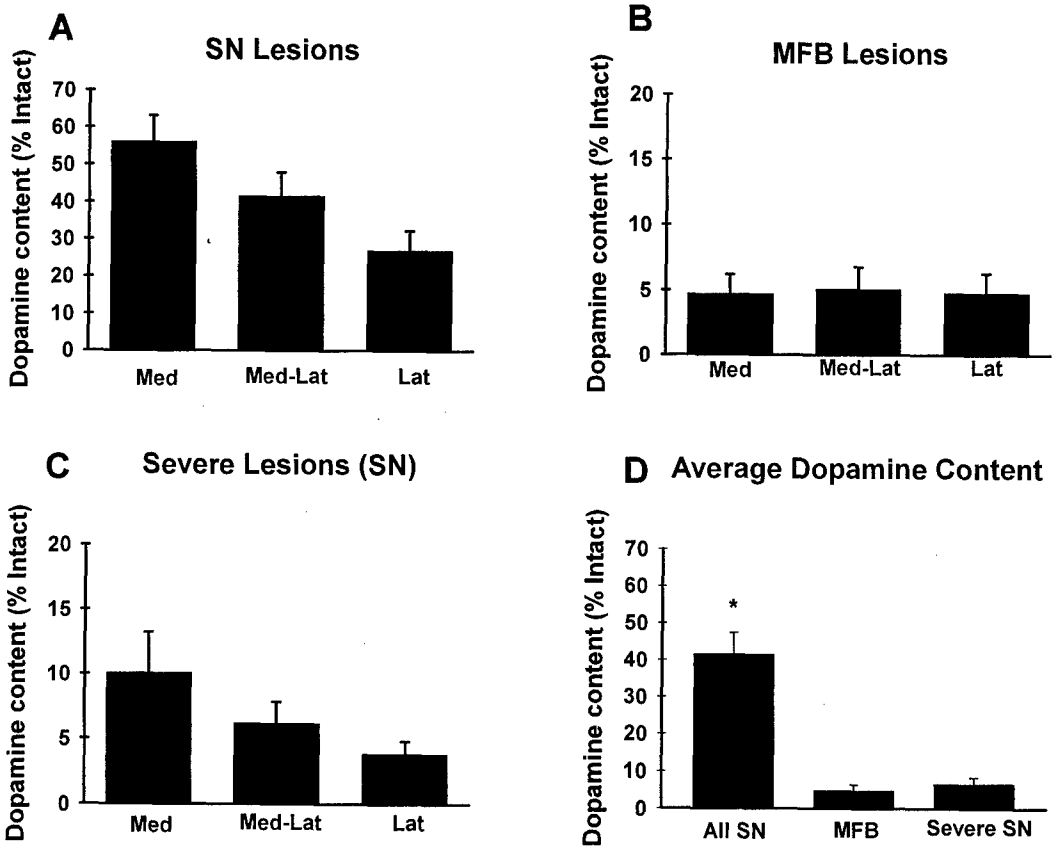
RESULTS

Lesions

Unilateral depletions of striatal dopamine were obtained by two different lesion methods: animals that received 6-OHDA injections into the lateral SN had graded lesions, and animals that received injections into the MFB had severe uniform depletions of striatal dopamine. Figure 1A shows the dopamine content of the medial, medio-lateral and lateral CP for all SN lesions. From the figure it appears that the lesion was graded, with lateral CP having the maximum dopamine depletion, the medio-lateral CP having intermediate depletion, and the medial CP having the least dopamine depletion, similar to our previous studies [52;115]. One-way ANOVA showed a significant difference between dopamine content of these three CP sub-regions ($p<0.01$, $F_{(2)}= 5.44$). The dopamine content of the CP sub-regions for animals with MFB injections is shown in Figure 1B. The depletion of dopamine was severe in these animals, and all three sub-regions showed similar levels of depletion. One-way ANOVA showed no significant differences between the dopamine contents of the CP sub-regions for the MFB lesions. To assess the differences between severe lesions obtained by MFB injection and severe lesions obtained by SN injection, a subset of the SN lesions that had severe depletions of striatal dopamine was analyzed separately. Figure 1C shows the dopamine content of the three CP sub-regions for animals with severe SN lesions. Since the neurotoxin was

injected in the lateral SN, these animals appear to have a trend of increasing depletion from the medial to the lateral CP. However, one-way ANOVA showed no significant differences between the dopamine content of the CP sub-regions for these animals. The average dopamine content of the CP for all SN lesions, for MFB lesions, and for the subset of SN lesions with severe dopamine depletion are plotted in Figure 1D. One-way ANOVA showed significant differences between the three groups ($p<0.001$, $F_{(2)}= 11.85$), and t-tests showed significant differences between the mean dopamine content of all the SN lesions, and the averages for the other two groups (MFB lesions and severe SN lesions). There was no significant difference between the average dopamine content of the severe MFB lesions and the severe SN lesions.

Fig.1. Pattern of CP denervation for SN and MFB lesions. Dopamine content of CP sub-regions for SN and MFB lesions is shown. Data are mean $\pm$ SEM. Abbreviations: SN = substantia nigra, MFB= medial forebrain bundle, Med = medial CP, Med-Lat = medio-lateral CP, Lat = lateral CP. (A) All SN lesions (n=29), significant difference between groups ($p<0.01$) by ANOVA. (B) Severe MFB lesions (n=11). (C) Subset of SN lesions with severe dopamine depletion (severe SN lesions, n=8). (D) Average dopamine content of the CP obtained for SN lesions, MFB lesions, and the severe SN lesions. * , significantly different from MFB lesions and severe SN lesions.


A
SN Lesions
Dopamine content (% Intact)
70
60
50
40
30
20
10
0
Med
Med-Lat
Lat
B
MFB Lesions
Dopamine content (% Intact)
20
15
10
5
0
Med
Med-Lat
Lat
C
Severe Lesions (SN)
Dopamine content (% Intact)
20
15
10
5
0
Med
Med-Lat
Lat
D
Average Dopamine Content
Dopamine content (% Intact)
*
70
60
50
40
30
20
10
0
All SN
MFB
Severe SN

Behavioral tests in substantia nigra lesions

Cylinder test

Asymmetry in forelimb use for vertical exploration along the wall of a cylinder was quantified by a single forelimb use asymmetry score, obtained by subtracting the percent use of the contralateral forelimb from that of the ipsilateral forelimb. These forelimb-use asymmetry scores were plotted against the dopamine content in the medial, medio-lateral and lateral CP (fig. 2 A, B, C respectively). For all sub-regions, there was a linear relationship of the asymmetry score with dopamine content ($p<0.01$), but this relationship was most prominent for the medial and medio-lateral CP ($r = 0.67$ for medial CP, $r = 0.68$ for medio-lateral CP, $r = 0.55$ for lateral CP). The pre-lesion average score falls on the regression line for the medial CP, but not for the other two sub-regions. For the lateral CP (Figure 2C), the dopamine content values were clustered at the more severe degrees of lesion, and the asymmetry scores appear to be relatively low until more severe lesion degrees were reached. Therefore, the distribution of the asymmetry scores appeared to indicate a curvilinear relationship between asymmetry scores and dopamine content. In accordance with this, application of a quadratic equation to this plot improved the r value ($r = 0.63$), but the improvement did not reach significance ($p = 0.0513$). The medio-lateral CP content has the greatest correlation with asymmetry scores (Figure 2B). However, like the lateral CP, it appears that the asymmetry scores are almost near baseline at mild and moderate lesion degrees, and increase only for more severe degrees of lesion. Again, application of a quadratic equation improved the r value to 0.72, but this improvement was not significant ($p = 0.07$). Similarly, application of a quadratic equation

to the plot of asymmetry scores for the medial CP increased the r value to 0.71 but failed to improve it to a statistically significant level ($p = 0.08$).

Figures 2 D, E, and F show the asymmetry scores of animals grouped by lesion degree based on dopamine content of the medial, medio-lateral and lateral CP respectively. When grouped based on dopamine content of the medial CP (Figure 2D), the asymmetry scores appear to be least during pre-lesion testing, greater for the mild lesions, still greater for the moderate lesions, and greatest for the severe lesions. One-way ANOVAs comparing these groups showed a significant effect of lesion ($p<0.001$, $F_{(3)}= 28.6$). On comparison with the pre-lesion score by t-tests, the increase in asymmetry was significant for the moderate ($p<0.05$) and severe lesions ($p<0.001$). The severely lesioned group was also significantly different from the moderately lesioned group ($p<0.01$). The difference between the pre-lesion and mildly lesioned groups did not reach significance. Figure 2E shows the asymmetry scores of the animals grouped into mild, moderate and severe lesion degrees based on dopamine content of the medio-lateral CP. Similar to the medial CP, one-way ANOVA showed a significant effect of lesion ($p<0.001$, $F_{(3)}= 25.97$) and t-tests demonstrated a significant difference between the pre-lesion score and the scores of the moderate ($p<0.05$) and severe lesion groups ($p<0.001$). Also, there was a significant difference between the moderate and severe lesion groups ($p<0.01$). Unlike the medial CP where the mildly lesioned animals had a greater score than pre-lesion values, these two groups had very similar mean scores for the medio-lateral CP. Figure 2F shows the asymmetry scores for animals grouped into different lesion degrees based on the dopamine content of the lateral CP. There was a significant effect of lesion by ANOVA ($p<0.001$, $F_{(3)}= 14.94$) and significant differences between the pre-lesion and

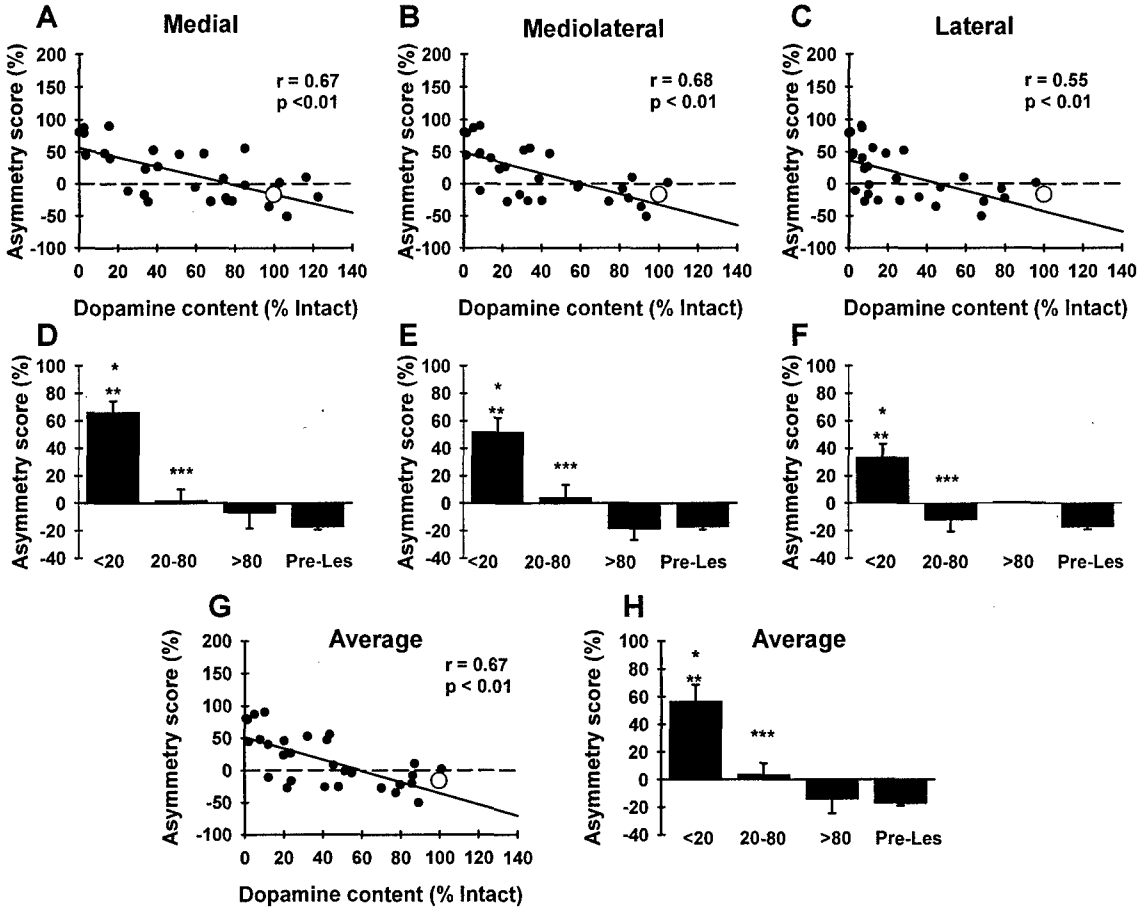
severe lesion groups ($p<0.001$). Also significant was the difference between the severely lesioned and moderately lesioned groups ($p<0.01$). Unlike for the medial and medio-lateral CP, there was no significant difference between the moderate lesions and pre-lesion groups. Also, the mildly lesioned group was not significantly different from the pre-lesion group. Therefore, for the lateral CP, the moderate, mild and pre-lesion groups were similar.

Figure 2G shows the individual asymmetry scores of the animals plotted against the average CP dopamine content. Like the three sub-regions, the average CP dopamine content was linearly related to the asymmetry scores ($r = 0.67$, $p<0.01$). Again, application of a quadratic equation improved the r value ($r = 0.72$) but not to a significant degree ($p = 0.07$). Figure 2H shows asymmetry scores of animals grouped into lesion degrees based on average CP dopamine content. One-way ANOVA showed a significant effect of lesion ($p<0.001$). Significant differences between the pre-lesion score and moderate ($p<0.05$) and severe lesions ($p<0.01$) were seen. Also, the moderate and severe lesion groups were significantly different from each other ($p<0.01$). The score of the mildly lesioned group was very similar to pre-lesion score.

Fig.2. Cylinder test. Panels A, B and C show the individual asymmetry scores of the rats with SN lesions against their dopamine content in the medial (A), medio-lateral (B), lateral (C) caudate-putamen. Panels D, E and F show the asymmetry scores of animals divided into severe (<20), moderate (20-80) and mild (>80) lesions based on the dopamine content of the medial (D), medio-lateral (E) and lateral (F) CP. Panel G shows the individual asymmetry scores plotted against the average dopamine content of the whole CP for the same animals. In panel H the animals are grouped according to average CP dopamine content. For panels D, E, F and H, data are mean $\pm$ SEM.

*, significantly different from pre-lesion score, $p<0.001$. **, significantly different from moderate lesions, $p<0.01$. ***, significantly different from pre-lesion score, $p<0.05$.

Abbreviations: <20= <20% dopamine content of intact side, severe lesions; 20-80= 20-80% dopamine content of intact side, moderate lesions; >80= >80% dopamine content of intact side, mild lesions; Pre-Les= pre-lesion scores


A
Medial
r = 0.67
p <0.01
B
Mediolateral
r = 0.68
p < 0.01
C
Lateral
r = 0.55
p < 0.01
Asymmetry score (%)
Dopamine content (% Intact)
D
E
F
<20
20-80
>80
Pre-Les
G
Average
r = 0.67
p < 0.01
H
Average

Vibrissae-evoked placing

Placing of forelimbs in response to stimulation of vibrissae was tested in three sessions, each on a different day. Data from the session with best placing performance (least number of unsuccessful placing) was used for analysis. Of the animals that showed placing deficits, none showed any recovery in the placing function on repeated testing over 1-2 weeks following lesion surgery. The method by which placing was elicited (direct placing, across-midline placing, and head-on placing) did not make a difference in the response obtained. Therefore, data from all three types of testing were pooled. With one exception, only animals with severe lesions showed deficits in placing (100% unsuccessful contralateral placing). The one animal that had a placing deficit but did not fall in the severe lesion group had a 100% unsuccessful score and an average dopamine content in the CP of 20.6%, with 51% intact innervation of the medial CP and severe lesions of the medio-lateral (8% intact) and lateral (2% intact) CP. Therefore, for the range of lesions obtained, most of the scores were at baseline (0 % unsuccessful contralateral placing), except at the severe denervation range (100% unsuccessful contralateral placing), and there were no scores in the intermediate range. This distribution of scores shows an abrupt increase from baseline at severe lesions, unlike the relatively smoother distribution of asymmetry scores on the cylinder test. Due to this clear division between animals that placed and did not place, linear regression was not performed.

Animals were grouped into mild, moderate and severe lesions based on average CP dopamine content, and the placing scores of the three groups are shown in figure 3A. Severe lesions show maximal deficits, with mild lesions and pre-lesion scores being zero.

The mean score of the moderate lesion was greater than zero because of the one animal mentioned earlier that had placing deficits. One-way ANOVA showed a significant effect of lesion ($p<0.01$, $F_{(3)}= 4.82$) on the placing response. Post-hoc t-tests showed that the mean score of the severely lesioned group was significantly different from mean pre-lesion score ($p<0.01$), whereas the mean score of the moderately lesioned group did not differ from the mean pre-lesion score. All animals (except one) with placing deficits had average CP dopamine contents that fell in the severe lesion range (<20% of intact side) and for these animals, the dopamine content of various CP sub-regions also fell within the severe lesion range. Therefore, sub-regional differences in CP dopamine content made little difference in the scores (data not shown).

Engagement and disengagement tests

Rats were placed in wire-mesh cages and their vibrissae touched with wooden probes during eating (disengagement test) and while not eating (engagement test). The shortest latencies to orient towards the probe, on the contralateral and ipsilateral side were recorded. For the disengagement test, none of the animals with mild and moderate lesions showed increased latency to respond (all responses within 1s). All the animals that showed an increased latency (disengagement deficit present) were severely lesioned. Since the latencies were not evenly spread out, linear regression was not performed. Animals were grouped into mild, moderate and severe lesions based on the CP average dopamine content. The contralateral latency to disengage from eating and respond to the probe for each of the three groups of animals and the latency during pre-lesion testing are shown in Figure 3B. One-way ANOVA showed a significant effect of lesion on disengage latencies ($p<0.001$, $F_{(3)}= 9.67$). The contralateral latency of the severely

lesioned group was significantly greater than that of the moderately lesioned group ($p<0.05$), and that the pre-lesion latency ($p<0.01$). Similar to our findings for the placing response, all animals that had disengagement deficits were severely lesioned in all three CP sub-regions. Therefore, grouping the animals by sub-region CP dopamine content made no difference in the distribution of disengagement latencies.

For the engagement test, all animals responded in less than 1 second. Linear regression did not show any significant correlation between latency to respond and CP dopamine content. Figure 3C shows the contralateral orienting latency for the engagement response. As shown in the figure, the latencies of all three lesion groups appear similar to pre-lesion controls. This was confirmed by one-way ANOVA which showed no significant effect of lesion on the contralateral orienting latencies for the engagement test. Orienting latencies on the ipsilateral side did not differ from pre-lesion values and between mild, moderate and severe lesion groups for both the tests (data not shown).

Passive initiation threshold (PIT) test

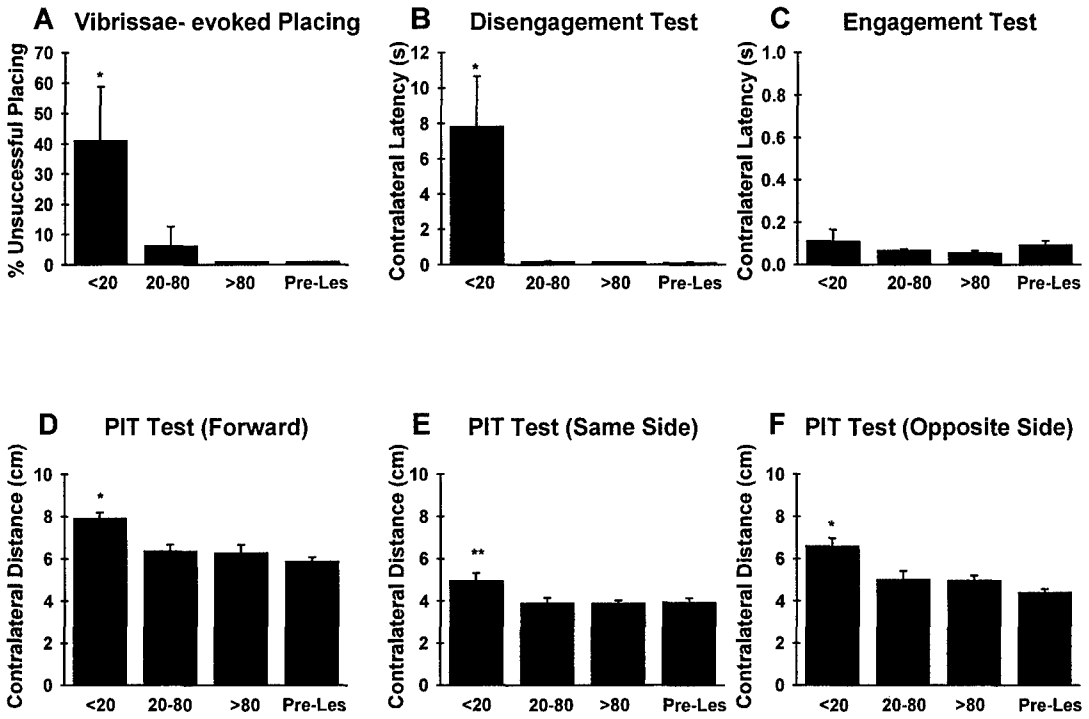
Animals were held in a way such that only one forelimb was allowed to be placed on the testing surface and were slowly moved forwards or sideways in either direction until the forelimb bearing weight stepped forwards. The distance covered was measured when each forelimb was bearing the animal's weight. For animals with severe lesions, the distance covered when the contralateral limb was bearing weight was increased. There did not appear to be an increase in distance for mild and moderate lesions over the pre-lesion score. Therefore, the relationship between PIT test scores and dopamine content was not smooth, and linear regression was not performed.

The distance covered when the contralateral forelimb was tested was averaged for each lesion group (mild, moderate and severe lesions) and plotted along with mean pre-lesion distances for the same forelimb in Figures 3D (forward PIT test), 3E (same side PIT test) and 3F (opposite side PIT test). As shown in the figures, an increase in distance was seen only in severely lesioned animals, for all methods of performing this test. This increase was seen in all the three different methods of doing the test. For all three methods, one-way ANOVA showed a significant effect of lesion ($p<0.001$, $F_{(3)}= 8.73$ for forwards PIT test, $p<0.05$, $F_{(3)}= 2.93$ for same side PIT test, and $p<0.001$, $F_{(3)}= 7.74$ for opposite side PIT test). Significant differences between the severely-lesioned group and pre-lesion mean score were seen on t-tests ($p<0.001$ for forwards PIT test, $p<0.05$ for same side PIT test, and $p<0.001$ for opposite side PIT test). For the forwards PIT test (Figure 3D), there were additional differences between the severely lesioned group and the other two groups ($p<0.05$ for mild lesions and $p<0.01$ for moderate lesions). When the test was done by moving the rats in the opposite direction as the weight-bearing forelimb, there was also a significant difference between the severely lesioned and mildly lesioned groups ($p<0.05$). Again, animals that showed an increase in distance were severely lesioned in all CP sub-regions, and grouping animals by sub-regional differences in dopamine content showed a similar distribution of scores as when grouping was done by average CP dopamine content (data not shown).

We tested the ipsilateral forelimb to see if there was any compensatory adaptation on the ipsilateral side, appearing as a shortening of the distance covered when compared to pre-lesion distance for the same limb. No such decrease in the distance traversed was seen. To test if this compensation could be detected by different methods of holding the

rats, and at different speeds of moving them, the test was repeated at a slower speed and with holding the rats at a greater angle to the horizontal plane, but shortening of ipsilateral distance was not detectable even in the severely lesioned group (data not shown). However, with all these different methods of doing the tests, the significant increase in contralateral distance for the severely lesioned group was maintained (data not shown).

Fig.3. Behavioral tests for SN lesions. Animals were grouped according to average CP dopamine content. Data are mean $\pm$ SEM. (A) Vibrissae-evoked placing. Y-axis shows % unsuccessful contralateral placing.*, significantly different from mean pre-lesion score, $p<0.01$. (B) Disengagement test. Y-axis is contralateral latency to disengage (s). *, significantly different from moderately lesioned group, $p<0.05$, and from pre-lesion latency, $p<0.01$. (C) Engagement test. Y-axis is contralateral latency to orient (s). (D) PIT test (forward), (E) PIT test (same side) and (F) PIT test (opposite side). Y-axis is distance covered when the contralateral forelimb bears weight. *, significantly different from pre-lesion distance, $p<0.01$. **, significantly different from pre-lesion distance, $p<0.05$. Abbreviations: <20= <20% dopamine content of intact side, severe lesions; 20-80= 20-80% dopamine content of intact side, moderate lesions; >80= >80% dopamine content of intact side, mild lesions; Pre-Les= pre-lesion scores

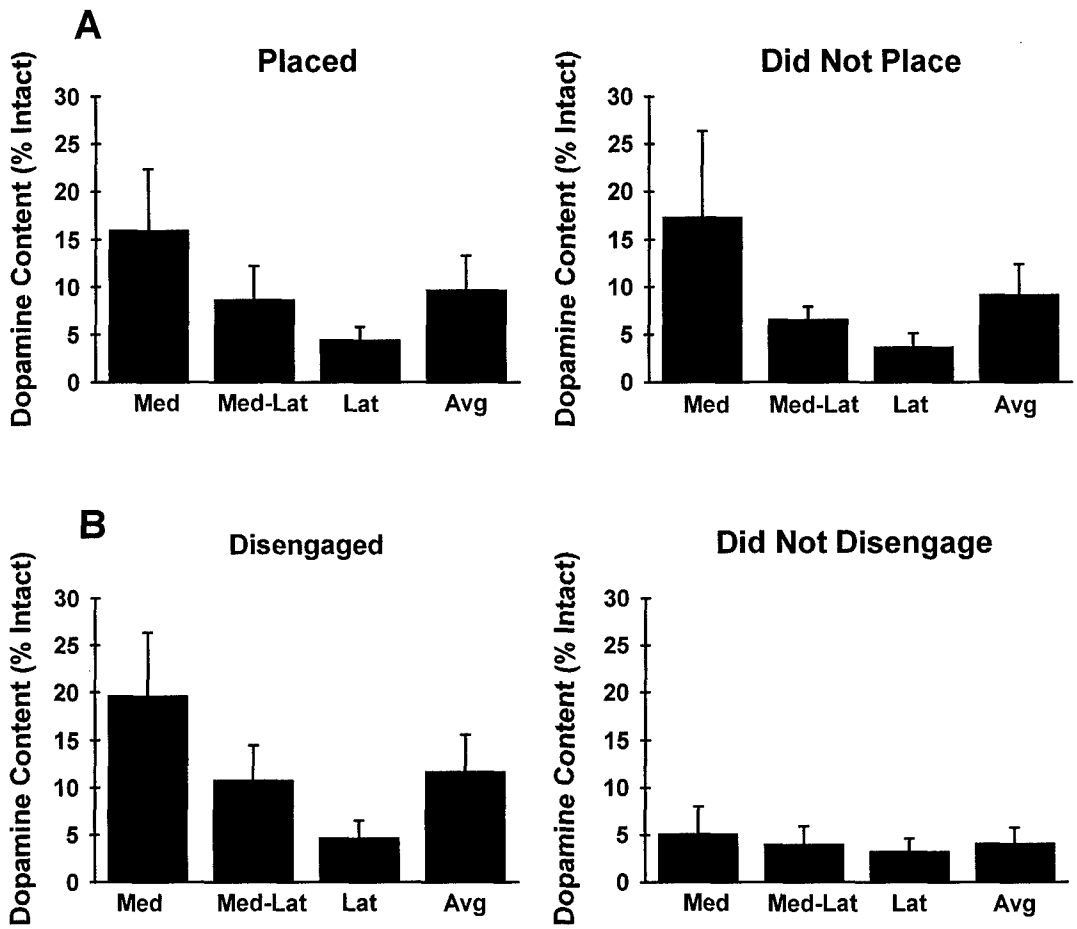

A Vibrissae- evoked Placing
% Unsuccessful Placing
70
60
50
40
30
20
10
0
*
<20
20-80
>80
Pre-Les
B Disengagement Test
Contralateral Latency (s)
12
10
8
6
4
2
0
*
<20
20-80
>80
Pre-Les
C Engagement Test
Contralateral Latency (s)
1.0
0.8
0.6
0.4
0.2
0.0
<20
20-80
>80
Pre-Les
D PIT Test (Forward)
Contralateral Distance (cm)
10
8
6
4
2
0
*
<20
20-80
>80
Pre-Les
E PIT Test (Same Side)
Contralateral Distance (cm)
10
8
6
4
2
0
**
<20
20-80
>80
Pre-Les
F PIT Test (Opposite Side)
Contralateral Distance (cm)
10
8
6
4
2
0
*
<20
20-80
>80
Pre-Les

Analysis of placing and disengagement response

For the vibrissae-evoked placing and disengagement tests, deficits were seen only in the severely lesioned group. However, there were some animals in this group which had no deficits at all, while others had maximal deficits. We hypothesized that this was probably because of sub-regional differences in CP dopamine content in these two subsets of animals, and these differences were examined by two-way ANOVA. The animals with average dopamine content of the CP less than 21% were divided into two groups based on their performance on these tests: those that had deficits, and those that did not. The cut off point was taken as 21% (as opposed to the 20% level used in all other data analysis for this study) since there was one animal in the moderately lesioned group which showed placing deficits, and its average CP dopamine content was 20.6%. This increase in the cut-off level for grouping added two more animals to the severe lesion group (average dopamine content 20.6% and 20.3%). Figure 4A shows the dopamine content of the CP sub-regions for animals that had no placing deficit (left panel) and those that had placing deficits (right panel). From the figure, the regional denervation pattern of the CP of both these subgroups appears similar. Two-way ANOVA comparing CP sub-region and response on the placing test showed a significant main effect of CP sub-region ($F_{(2,24)}= 3.63$, $p<0.05$), but not of placing response. The average CP dopamine content for the two groups is very similar and the interaction between sub-region and placing response was not significant. Therefore, the difference in placing response within the severely lesioned animals is not explained by sub-regional innervation differences, and the reason for this differential response is not known.

Figure 4B shows the dopamine content of the CP sub-regions for animals that had no deficit in the disengagement response (left panel) and those that did have a disengagement deficit (right panel). From the figure it appears that animals with deficits in disengagement had lesser dopamine content in the CP (especially the medial CP) than animals that had no deficits. Two-way ANOVA comparing CP sub-region and response on the disengagement test showed a significant main effect of the disengagement response ($F_{(1,18)}= 6.92$, $p<0.05$), but no significant main effect of CP sub-region or interaction of the sub-region and disengagement response. Thus the average dopamine content of the animals that had no deficit is greater than that of animals that had a disengagement deficit. Also, there seemed to be a trend for animals with deficits to be uniformly lesioned throughout the CP, unlike those with no deficits which were graded lesions. Therefore, sub-regional differences in dopamine content, especially in the medial CP, may play a role in the disengagement function.

Fig.4. Analysis of placing and disengagement tests. Dopamine content of medial, medio-lateral and lateral CP and average CP dopamine content is plotted. Data are mean $\pm$ SEM. (A) Vibrissae-evoked placing test. Left panel shows data from animals that had no placing deficits. Right panel shows data from animals that had placing deficits. Two-way ANOVA showed a significant main effect of CP sub-region, $p<0.05$. (B) Disengagement test. Left panel shows animals that had no disengagement deficits. Right panel shows data from animals that had disengagement deficits. Two-way ANOVA showed a significant main effect of the disengagement response, $p<0.05$. Abbreviations: Med = medial CP, Med-Lat = medio-lateral CP, Lat = lateral CP, Avg= average CP dopamine content

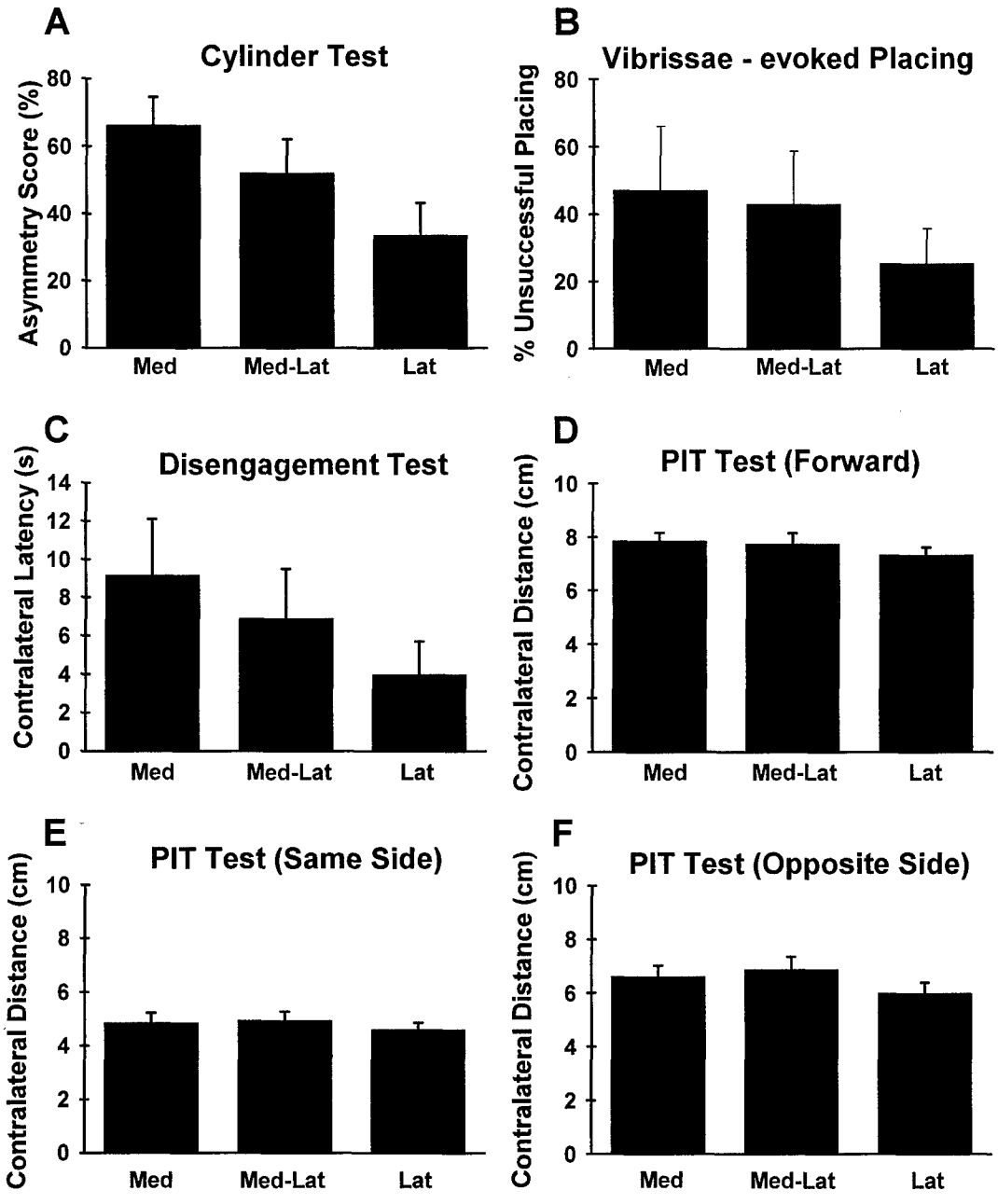

A
Placed
Did Not Place
B
Disengaged
Did Not Disengage
Dopamine Content (% Intact)
0
5
10
15
20
25
30
Med
Med-Lat
Lat
Avg

Severe SN lesions

The graded SN lesions show increasing dopamine depletion from the medial to the lateral CP. For an animal that has a severe depletion in the lateral CP, the degree of denervation may fall within the mild or moderate depletion range in the medial and medio-lateral CP. Therefore, in an animal with severe denervation of the lateral CP, any behavioral deficits may be attributed either to the severe depletion in the lateral CP or to lesser denervations in the medial and medio-lateral CP. In order to study the behavioral effects of severe dopamine depletion in each CP sub-region, the animals which had severe denervations of the medial, medio-lateral and lateral CP (dopamine content < 20% of control side for each sub-region) were selected, and the test scores for each group of severely lesioned animals was plotted (Figure 5). The scores on the cylinder test (Figure 5A), vibrissae-evoked placing (Figure 5B) and disengagement tests (Figure 5C) were lowest for those with severe lesions of the lateral CP, higher for those with severe lesions of the medio-lateral CP, and highest for the group with severe lesions of the medial CP. However, these differences were not significant by one-way ANOVA. For the PIT test (fig.6D-F), the scores for the rats with severe lesions in the three sub-regions were almost equal. Although the sub-regional differences in severe denervation do not have a statistically significant effect on the behaviors evaluated, there was a trend for severe lesions in the medial CP to have the greatest effect on these behaviors, followed by severe lesions of the medio-lateral CP and then the severe lesions of the lateral CP, for the cylinder, placing and disengagement tests. Perhaps a finer measurement of regional innervation such as by using the punch technique [120] will identify more precisely the

relationship of severe sub-regional dopamine depletion and deficits on these behavioral tests.

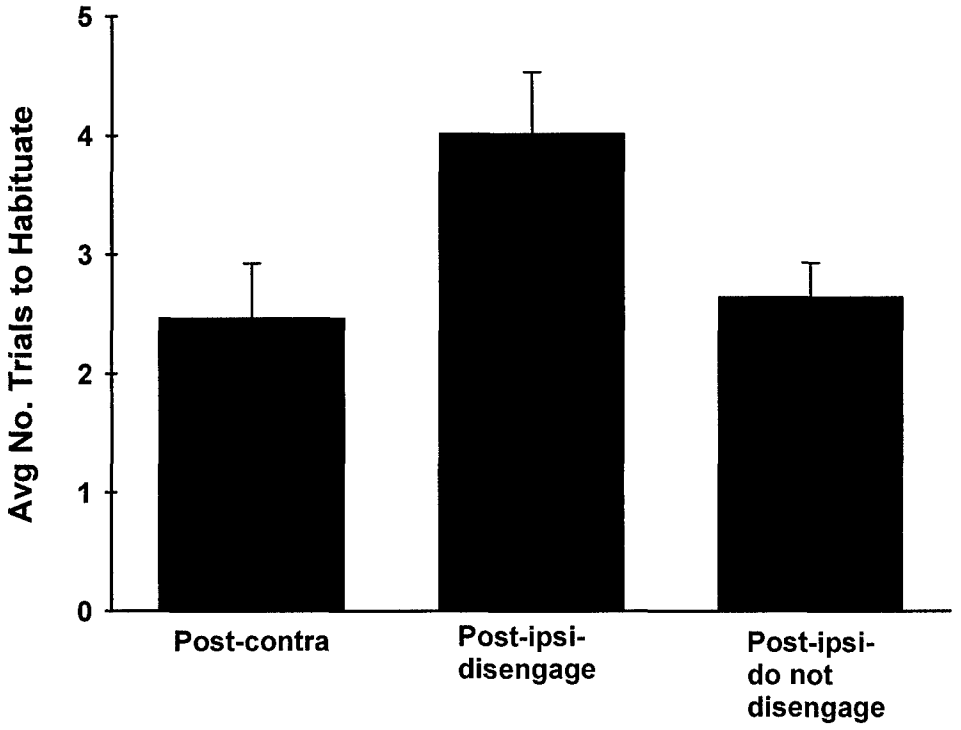
Fig.5. Effect of severe lesions of CP sub-regions. Animals with less than 20% dopamine innervation remaining in the medial , medio-lateral and lateral CP grouped together and scores are plotted for each group. Data are mean $\pm$ SEM. (A) Cylinder test. Y-axis shows % limb use asymmetry. (B) Vibrissae-evoked placing. Y-axis shows % unsuccessful placing by contralateral limb. (C) Disengagement test. Y-axis shows latency to disengage (s). (D), (E), (F): PIT test, forwards, same side, and opposite side respectively; Y-axis shows distance (cm) covered when contralateral forelimb bears weight. Abbreviatons: Med = medial CP, Med-Lat = medio-lateral CP, Lat = lateral CP


A
Cylinder Test
Asymmetry Score (%)
0
20
40
60
80
Med
Med-Lat
Lat
B
Vibrissae - evoked Placing
% Unsuccessful Placing
0
20
40
60
80
Med
Med-Lat
Lat
C
Disengagement Test
Contralateral Latency (s)
0
2
4
6
8
10
12
14
Med
Med-Lat
Lat
D
PIT Test (Forward)
Contralateral Distance (cm)
0
2
4
6
8
10
Med
Med-Lat
Lat
E
PIT Test (Same Side)
Contralateral Distance (cm)
0
2
4
6
8
10
Med
Med-Lat
Lat
F
PIT Test (Opposite Side)
Contralateral Distance (cm)
0
2
4
6
8
10
Med
Med-Lat
Lat

Habituation

The sub-group of animals in which habituation of disengagement response was tested was additionally divided into two groups: those that disengaged within 15 seconds in post-lesion testing and those that did not. For those that did disengage, number of trials to habituate on both ipsilateral and contralateral sides was recorded. For animals that failed to disengage, only the number of trials to habituate on the ipsilateral side could be recorded. The number of trials for the habituation of the disengagement response for these three groups are shown in Figure 6. It appears from the figure that habituation takes more trials on ipsilateral stimulation in animals that had no disengagement deficits than the other two groups which both appear to take a similar number of trials to habituate as each other (contralateral side in animals with no disengagement deficits, and ipsilateral side in animals with contralateral disengagement deficits). One-way ANOVA was significant for these three groups ($p<0.05$, $F_{(2)}= 2.49$), but post-hoc t-tests showed no significant differences between the groups. The average number of trials to habituate on the ipsilateral and contralateral sides did not significantly differ from each other during the pre-lesion testing period (data not shown).

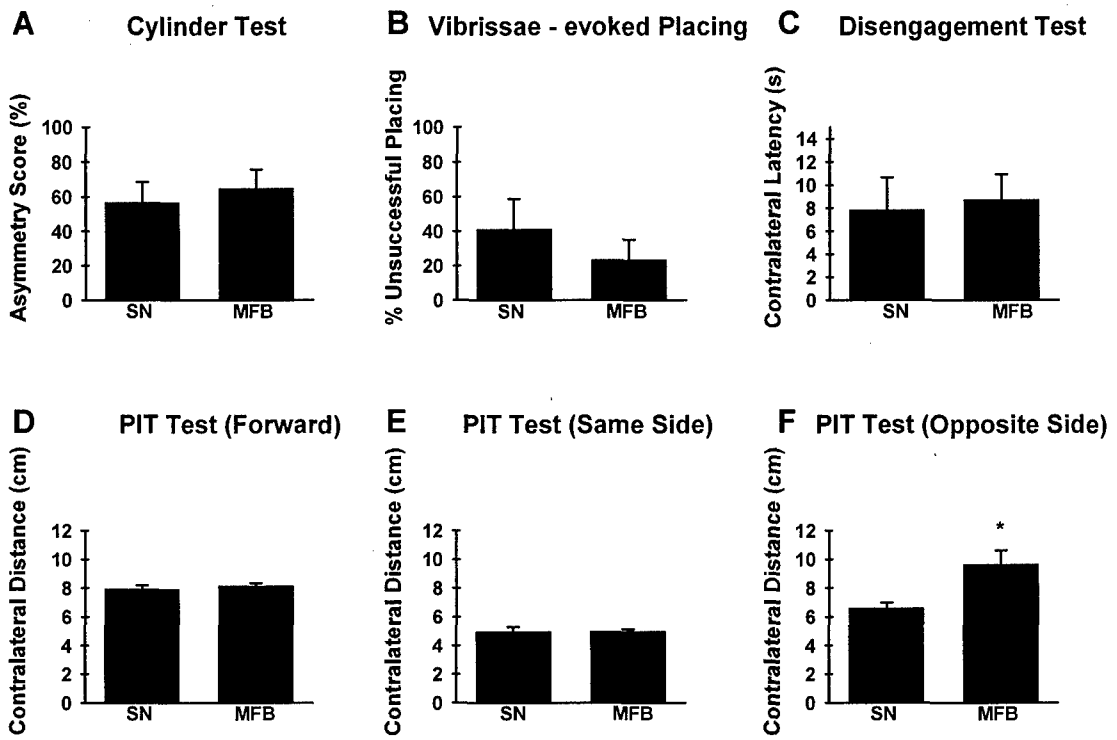
Fig.6. Habituation of disengagement response. Number of trials for habituation of disengagement response plotted. Data are mean $\pm$ SEM . Bars represent, from left to right, contralateral side in animals that had no disengagement deficit (post-contra), ipsilateral side in animals with no disengagement deficit (post-ipsi-disengage) and ipsilateral side in animals that failed to disengage (post-ipsi-do not disengage).


Avg No. Trials to Habituate
5
4
3
2
1
0
Post-contra
Post-ipsi-disengage
Post-ipsi-do not disengage

SN vs MFB lesions

One goal of this study was to compare behavioral deficits obtained by graded SN lesions with severe dopamine depletion to those obtained by severe uniform MFB lesions. A subset of animals with SN lesions that had severe depletions of striatal dopamine was chosen, and their performance on the behavioral tests was compared to that of severe lesions obtained by 6-OHDA injections into the MFB. Figure 1 shows that the average CP dopamine content of these two groups was not statistically different from each other. Figure 7 shows the average scores (±SEM) of the severe SN lesions and the severe MFB lesions on the cylinder test (Figure 7A), vibrissae-evoked placing (Figure 7B), disengagement test (Figure 7C), and the three types of the PIT test (Figure 7D-F). On comparison of behavioral test scores of these two lesion types using t-tests, there was no significant difference between the scores on all tests except the sideways PIT test with the rats being moved in the opposite direction as the forelimb bearing weight (Figure 7F, $p<0.05$). This shows that, in general, the severe SN lesions are comparable to the severe MFB lesions in their performance in most of these tests.

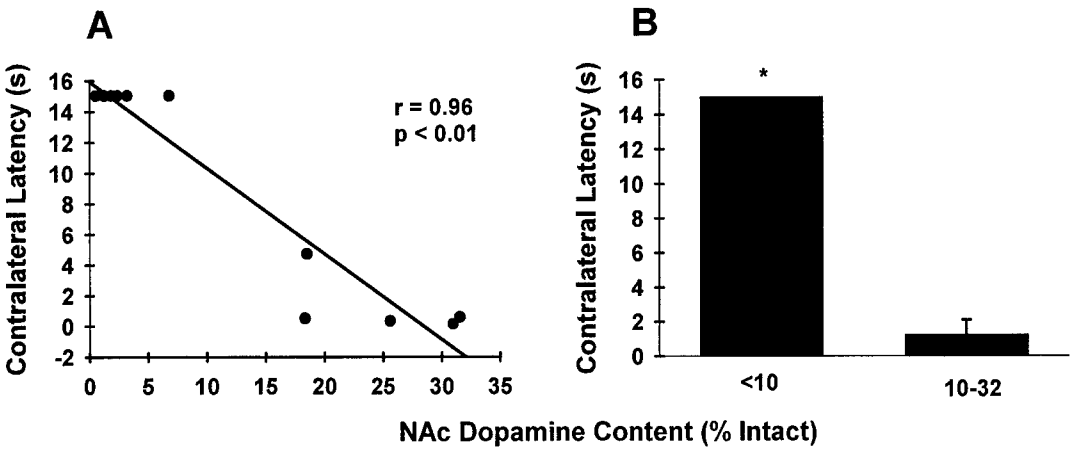
Fig.7. Comparison of behavioral deficits produced by severe SN and MFB lesions. Data are mean $\pm$ SEM. (A) cylinder test (B) vibrissae-evoked placing (C) disengagement test (D) PIT test (forwards) (E) PIT test (same side) (F) PIT test (opposite side). *, significantly different from mean score of severe SN lesions, $p<0.05$. Abbreviations: SN= substantia nigra, MFB= medial forebrain bundle


A
Cylinder Test
Asymmetry Score (%)
100
80
60
40
20
0
SN
MFB
B
Vibrissae - evoked Placing
% Unsuccessful Placing
100
80
60
40
20
0
SN
MFB
C
Disengagement Test
Contralateral Latency (s)
14
12
10
8
6
4
2
0
SN
MFB
D
PIT Test (Forward)
Contralateral Distance (cm)
12
10
8
6
4
2
0
SN
MFB
E
PIT Test (Same Side)
Contralateral Distance (cm)
12
10
8
6
4
2
0
SN
MFB
F
PIT Test (Opposite Side)
Contralateral Distance (cm)
*
12
10
8
6
4
2
0
SN
MFB

Effect of severe NAc depletions in MFB lesions

A previous study had shown a linear relationship between disengagement latency and nucleus accumbens (NAc) dopamine content, at severe degrees of dopamine depletion in the NAc [121]. In order to examine the effects of severe nucleus accumbens dopamine depletion on the behaviors evaluated here, we determined NAc dopamine content for the severe MFB lesions (average CP dopamine content < 20%). All severe MFB lesions had NAc dopamine content less than 32% of the intact side. The disengagement latency was plotted against NAc dopamine content (Figure 8A). Linear regression showed a significant correlation between disengagement latencies and NAc dopamine content ($r=0.96$, $p<0.01$). However, the latencies for these animals appeared clustered at two levels: those animals with very severe depletions of the NAc dopamine (<10% of intact side) showed increased latency while animals with 10-32% dopamine content intact showed no deficits. Therefore, the animals were divided into two groups: those whose NAc dopamine content was less than 10% and those whose NAc dopamine content was 10-32% of the intact side. The disengagement latencies of these two groups is shown in Figure 8B, and the t-test showed a significant difference between these groups ($p<0.001$). For all the other behavioral tests, the relationship of test scores to NAc dopamine content was not linearly related (data not shown).

Fig.8. Disengagement test: effect of NAc dopamine depletion. Data from animals with MFB lesions shown. (A) Latency to disengage (s) shown on Y-axis. (B) Latency to disengage (s) in animals with very severe depletions of NAc dopamine (<10% of intact side) and less severe depletions (10-32% of intact side) shown on Y-axis. Data are mean $\pm$ SEM. *, significantly greater than 10-32% intact group, $p<0.001$. Abbreviations: NAc= nucleus accumbens, <10= <10% dopamine content of intact side, 10-32= 10-32% of dopamine content of intact side


A
B
Contralateral Latency (s)
16
14
12
10
8
6
4
2
0
-2
0
5
10
15
20
25
30
35
r = 0.96
p < 0.01
*
<10
10-32
NAc Dopamine Content (% Intact)

CHAPTER V

DISCUSSION

The main goal of this study was to identify behavioral deficits that are linearly related to CP dopamine content in animals with a full range of dopamine denervation using a graded SN lesion model. A behavior with such a relationship to dopamine content could be mediated by phasic dopaminergic signaling. This graded lesion has been used by us previously for microsensor experiments [115], and the effect of such a lesion on behavior was not known. The major conclusion of this study is that the behavior measured on the cylinder test is linearly related to the degree of dopamine innervation in the striatum while all other tests evaluated show deficits only in severely lesioned animals.

Lesions

In agreement with our previous work [52;115], injection of 6-OHDA into the lateral SN causes graded lesions of the CP, with a greatest denervation in the lateral CP, lesser in medio-lateral CP and least in the medial CP. On the other hand, injections into the MFB are more uniform and severe. Severe lesions caused by SN injections do show a trend towards being graded, but this difference in the innervation of the CP sub-regions was not significant. This is probably because at more severe degrees of neuronal loss, all the CP sub-regions have dopamine contents in the severe-lesion range, and there are only very small differences between sub-region innervation degrees. Therefore, although sub-

regional differences in innervation do occur in severe graded SN lesions, they are not as pronounced as in mild and moderate lesions. The average dopamine content of the severe SN lesions is similar to that for MFB lesions. These two lesion types are therefore comparable in terms of overall dopamine depletion degree.

Cylinder test

Of the behavioral tests evaluated, only the cylinder test allowed detection of asymmetry at partial degrees of dopamine denervation. This is the first report of a linear relationship of dopamine content and asymmetry scores on the cylinder test. Significant differences between the mean scores of severely lesioned animals (<10% intact) and moderately lesioned animals (30-70% intact) from each other and from the pre-lesion mean score have been shown previously in rats with MFB and striatal lesions [53].

The CP is known to have sub-regional differences in innervation and behavioral functions. In this study, the medial and medio-lateral CP dopamine content appeared to have a linear relationship with scores on the cylinder test, while the relationship was more curvilinear for the lateral CP. The pre-lesion mean score falls on the regression line in the medial CP unlike for the lateral CP. Another reason to support a linear relationship for the medial and medio-lateral CP is that improvement in the r value with a curvilinear plot fit (quadratic equation) was less significant for these two sub-regions than that for the lateral CP ($p= 0.08$ for medial CP, $p=0.07$ for the medio-lateral CP, and $p=0.051$ for lateral CP). Also, plots showing asymmetry scores when animals were grouped by dopamine content show that for the medial CP, the asymmetry scores increase sequentially from the pre-lesion score to the mild, moderate and severe lesions. Although there is no significant increase in scores of the mild lesions when compared to pre-lesion

score, there is a definite trend. For the medio-lateral CP, the mildly lesioned animals have a score similar to pre-lesion score, but there is a trend towards increasing asymmetry from the mild to the severe lesions. For the lateral CP, the scores for even the moderate lesions are similar to pre-lesion scores, and the only group exhibiting asymmetry appears to be the severely lesioned animals. Therefore, the dorsal medial and medio-lateral CP dopamine content, being linearly related to asymmetry scores, seem to be the best predictors of performance on the cylinder test. If the relationship were curvilinear, the lateral CP shows the best correlation of asymmetry scores with dopamine content.

In contrast to the implication of the medial CP for asymmetry of forelimb use in the cylinder test, studies of partial striatal 6-OHDA and ibotenic acid lesions have shown that the lateral striatum is important for various sensorimotor behaviors including forelimb use. Lesions of the lateral striatum or the ventro-lateral striatum, but not the medial striatum, cause deficits in forepaw use that manifest as difficulty in holding and manipulating food pellets, and performance of skilled forepaw reaching tasks [69;74;76-78]. Forelimb use for stepping was seen to be reduced in animals with lesions of the lateral and ventral parts of the striatum but not of the dorsomedial parts [79]. Transplants of embryonic substantia nigra grafts so as to re-innervate the ventrolateral striatum in 6-OHDA lesioned animals improve performance on certain tests of limb use, whereas grafts innervating the dorsal striatum do not [80].

The medial striatum, on the other hand, seems to have a role in spontaneous and drug-induced rotation, and in general levels of locomotor activity [72;99]. It also seems to be important for spatial tasks and for learning [95;97]. In this context, one would expect that the use of forelimbs in the cylinder test may be mirrored by the dopamine

content in the lateral CP, but our findings are contrary to this expectation. However, the cylinder test may not be a measure of skilled forepaw use, but is said to be an equivalent of postural instability in the unilateral lesion model of PD in rats. Postural instability in human patients is said to be due to involvement of axial structures, and not the limbs [122;123]. Levodopa-induced abnormal involuntary movements involving axial structures such as the trunk, head and neck occur in animals with lesions of medial CP but not in animals with CP lesions sparing this region [124]. Also, injection of amphetamine into the medial parts of the CP produces an increase in general locomotor activity and rearing behavior which involve use of axial musculature, while injection into the lateral CP does not [83]. Therefore, it appears that axial structures may be controlled by the medial part of the CP. If the cylinder test is a measure of postural instability and scores depend on involvement of axial musculature and not of the forelimbs, the occurrence of a linear relationship with dopamine content of the medial CP and absence of a linear relationship with lateral CP dopamine content could be explained. Another possibility is that this relationship of the cylinder test asymmetry to medial CP dopamine content could be an artifact of the graded lesion model used. All animals had more severe lesions of their lateral CP than the medial CP. Therefore, the true relationship of cylinder test deficits to dopamine content may be that reflected by the lateral CP (curvilinear relationship), although the linear correlation with medial CP dopamine is better.

Vibrissae-evoked placing test

Vibrissae-evoked placing performance is not linearly related to degree of lesion, but deficits appear only in the most severely lesioned animals. The vibrissae-evoked placing response was elicited by three different methods: direct, across-midline, and

head-on placing. Across-midline placing tests for sensory impairment in the contralateral vibrissae. If contralateral vibrissae can elicit placing in the ipsilateral forelimb but not in the contralateral forelimb, then sensory impairment can be ruled out. The head-on placing test was done to detect appearance of any recovery of the placing function in rats that showed initial deficits. The responses of all animals were similar for all three methods of doing the test; which means that no animal had sensory deficits, and none showed any recovery from placing deficits. Testing was carried out on three separate days, and some animals had higher scores (decreased number of placing) on some testing sessions compared to other sessions. These animals could have poorer performance on some testing sessions probably due to distraction on their part or some variation in the way that they were held during testing on that particular test day. The day when the placing deficits were minimal was chosen for analysis because that is the best performance the rats were capable of.

In our study, placing deficits appeared only in severely lesioned animals. In most other studies, this test had been performed only in severe lesions [54;55]. In one study, placing deficits appeared in moderately lesioned rats (mean total striatal dopamine content 33% of control values, ventrolateral striatum content 18% of control, and mediodorsal content 49% of control) [59]. However, the lesion method used in this study was injection of 6-OHDA into the ventrolateral striatum, whereas our lesion model involves injection of the neurotoxin into the lateral SN. Also, the animals in the above study had higher dopamine contents than the severely lesioned animals in our study in the portions of the CP besides the ventrolateral part. Such a difference in regional innervation could also contribute to the different results obtained. Even among the severely lesioned

animals in our study, only some showed deficits in placing. Among animals with severe lesions, comparison of the CP dopamine content of those that had deficits in placing with those that did not have deficits showed no significant difference between the groups. This could be because we did not identify antero-posterior differences in CP denervation, and such differences could play a role in placing behavior. Finer dissection methods to detect differences between smaller sections of the CP could pin-point the CP sub-region responsible for this behavior. Another possibility is that placing deficits may not be entirely mediated by dopaminergic deficits in these animals, but differential reduction in some other neurotransmitter may be also involved.

Engagement test

For all degrees of lesion and both types of lesion, our study showed no significant increase in latency to orient for the engagement test. This finding is similar to that seen in some previous studies [56;125;126]. However, some studies did show a significant increase in contralateral latency to respond in severely lesioned animals [57;93;121], and in one study there was an increased latency to respond on both sides in unilaterally lesioned animals, but the latencies on the contralateral side were greater [127]. In this last study, testing was conducted on four consecutive days following lesion surgery, although it is unclear how many days after the surgery the first testing session was conducted. If the testing was done on the day after the lesion, slowing of response time could be explained by the fact that the animals probably had not recovered from the initial effects of the surgery. In one study that showed increased latency [57], the increase was seen only for a few months after lesion surgery, and latency eventually returned to pre-lesion levels. In our subjects, we did notice some slowing in the orienting response in some

severely lesioned animals, but this slowing was seen only in some trials. All the previous studies except one [126] were conducted in severely lesioned animals. The range of partial lesions was limited in the one study in which partially lesioned animals were tested (only 4 animals with < 70% loss of SN tyrosine hydroxylase positive neurons) [126]. Therefore, we tested the whole range of lesion degrees and conclude that there is no delay in the engagement response for all degrees of lesion tested.

Disengagement test

We show that the disengagement latencies of the severely lesioned animals were significantly increased above the pre-lesion latencies. The deficit is not linearly related to the degree of lesion. This is similar to findings of other studies [56;57;93;121;126;128]. All these studies except one were done on animals with severe lesions. The study in which a range of lesions was tested [126] had similar results as our study (in that the disengage deficit appeared at over 80% dopamine loss in the striatum), but the range of partial lesions was limited, and there were only 4 animals with <70% depletion of tyrosine hydroxylase positive neurons in the SN. Another study showed increased latency to respond on both sides in unilaterally lesioned animals [127], but as mentioned in the previous section (under the engagement test), this may be because testing was done the days immediately following 6-OHDA injection, before the animal recovered from the surgery.

It has been shown that the disengage deficit is proportional to NAc dopamine content in animals with severe depletions of NAc dopamine that had grafts of nigral cell suspensions in their striatum [121] . We found a similar relationship between dopamine content of the NAc and the disengage latency. In our study, testing was stopped if the

animals did not respond within 15 seconds, and therefore for animals that did not disengage, the maximum time recorded was 15 seconds. In the previous study mentioned above, testing was conducted for a longer time (180 seconds), and individual differences in latency for animals that displayed a disengagement deficit were detected. The range of NAc lesions tested in the previous study was narrow and in the very severe depletion range (>96% lesioned). All MFB lesioned animals that had disengagement deficits in our study had NAc dopamine content that was close to this severe range. Therefore, in our study, a plot of disengagement latency against NAc dopamine content shows a clustering of scores at 15s for animals that have deficits, and near baseline for animals that did not show deficits. This ceiling effect of the duration for which testing was conducted prevented us from exploring individual differences in disengagement response for animals that showed deficits. In order to show the difference in disengagement response with different degrees of NAc dopamine depletion, the animals were divided into two groups: those that had lesioned NAc dopamine content <10% of the intact side and those that had 10-32% of dopamine content of intact side. The comparison between these two groups clearly brought out the significance of very severe depletions of NAc dopamine for the occurrence of disengagement deficits.

Habituation of the disengagement response on repeated testing was another aspect of the disengage deficit that we studied. In animals that failed to disengage from eating when vibrissae were stimulated on the side contralateral to the lesion, there was an anecdotal observation that the ipsilateral side did not habituate to repeated testing. This was unlike non-lesioned animals that stop responding after a few trials. It was expected that the average number of trials to habituate on the ipsilateral side would be much

greater in animals that did not disengage on the contralateral side than in animals that were not lesioned or did not show a disengage deficit. However, we did not observe such findings in our study. In fact, the animals that did not show a disengage deficit took a greater number of trials to habituate on the ipsilesional side than animals that did show a deficit in disengagement, although this difference was not significant. Also, for animals with no disengagement deficit, the number of trials to habituate was greater for the ipsilateral side than the contralateral side. Therefore, although we did not see a deficit in habituation in severely lesioned animals, there were interesting unexplained differences between the ipsilateral and contralateral sides for animals that did not show a disengagement deficit.

PIT test

For all the different ways in which the PIT test was performed (forward, sideways –same side and sideways-opposite side), there was a significant increase in contralateral scores for the severely lesioned group. However, unlike the findings of another group that was evaluating this test, shortening of the ipsilateral distance compared to pre-lesion values was not seen for any group [118]. This could be explained by differences in the method of holding the rats and the speed at which the testing was done. Six rats with severe SN lesions and 11 rats with severe MFB lesions were tested at both slow and fast speeds and while holding the rats at two different angles. However, even this failed to show any significant shortening of ipsilateral scores in all of the tests. One reason for this discrepancy could be that in our study, the post-lesion behaviors were recorded two weeks after lesioning, which may not be sufficient time for behavioral compensation to appear on the side ipsilateral to the lesion. Also, there was a roughly 100g weight gain in

our animals when post-lesion testing was done, compared to pre-lesion testing days. This increase in size could lead to the ability to cover longer distances before the center of gravity moves out of the base of the body for the larger rats, causing them to step forwards to regain balance after they have been moved for a longer distance. Hence, post-lesion shortening of distance covered when the ipsilateral forelimb bore weight may have been masked by this natural increase in tolerance to weight shift. We do not know if the other group that evaluated this test had any difference in the size of animals between the test and control groups. The validity of this argument could be tested by performing the PIT test on animals that are older and have established a steady weight before lesioning.

Comparison of severe SN lesions with MFB lesions

The performance of the animals with severe graded SN lesions was very similar to that of animals with severe MFB lesions for most of the tests. In one form of the PIT test (moving the rats in the opposite direction as the weight-bearing forelimb) the MFB lesioned group had a greater score. This could be because the average dopamine content of the MFB lesions was less than that of the severe SN lesions, although that difference did not reach significance. Therefore, severe graded lesions made by injection of 6-OHDA into the lateral SN are not different from the severe MFB lesions in terms of the behavioral deficits caused. The behavioral correlates of the whole range of lesions obtained by SN injections are therefore also comparable with behavior in MFB lesioned animals with partial degrees of neuronal loss.

Significance

Phasic dopaminergic signaling is said to be important for various aspects of behavior. According to Schultz et al., phasic dopamine signaling encodes error in reward

prediction [101]. For example, phasic activation occurs when a reward is unexpected or secondary reinforcers are presented before the reward. There is a decrease in activation when the expected reinforcement fails to occur, or when behavioral errors are made. However, Redgrave et al. [114] have proposed that phasic activation is important for "behavioral switching", and is essential for switching attention and behavior in response to salient stimuli in the environment. An increase in the number of dopamine concentration spikes, a component of phasic signaling, has been detected in rats during exposure to a novel environment, during sexual behavior, and when conspecifics are introduced into the testing area [111-113]. Unlike dopaminergic tone, there is some evidence that phasic signaling may not be maintained when dopamine depletion occurs. In one study using fast scan cyclic voltammetry, a decrease in the amplitude of electrically-evoked concentration spikes of dopamine (that may mimic naturally occurring dopamine transients) was seen with increasing dopamine depletion of the striatum [115]. This could mean that behaviors mediated by phasic signaling are not maintained when dopaminergic neurons are lost, and deficits appear when the neuronal loss is moderate or even mild. A behavior where deficits are linearly related to striatal dopamine content, and mirror the decrease in phasic signaling with increasing denervation may be one that is mediated by phasic signaling. From this study, it can be said that the behavior measured on the cylinder test (postural instability) may be mediated by a reduction in phasic dopaminergic signaling.

Since there is a reduction in amplitude of electrically-evoked dopamine transients in animals with partial dopamine depletion, we can hypothesize that a reduction in phasic signaling occurs early on in the neurodegenerative process of PD. Some studies in animal

models of the PD have shown that forced physical activity of affected limbs, started immediately after injection of a neurotoxin into the substantia nigra, can be protective against development of both neurochemical changes and symptoms of PD [13;129]. Such protection is probably related to exercise-induced production of GDNF in the striatum [12]. An implication of these findings is that physical therapy started early in the disease process may prevent or slow disease progression. Moreover, treatment with neuroprotective agents may soon be a possibility, and such treatments will particularly benefit patients in the pre-clinical and early stages of PD. Therefore, early diagnosis of the disease is especially important. By determining behaviors that are related to phasic signaling (which putatively diminishes with increasing degeneration of nigrostriatal dopamine neurons) in animal models of PD, we can obtain a better understanding of the changes that occur during the pre-clinical phase. This may set the stage for the development of methods for early diagnosis of PD. We have shown here that the cylinder test, which is a measure of postural instability, shows a linear relationship with CP dopamine content. Therefore, testing for postural instability may aid in early diagnosis of PD.

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Orienting and Placing

12

TIM SCHALLERT AND MARTIN T. WOODLEE

It is not difficult to document the existence of sensory or motor function asymmetries in normal rats or rats with unilateral damage to the basal ganglia, sensorimotor cortex, and related systems throughout the central nervous system, especially when the deficit on one side is near maximal. When the unilateral deficit is subtotal, quantifying the extent of asymmetry and changes over time requires unique testing methods that directly pit one hemisphere against the other.

As an illustration, if a person is slightly hard of hearing in one ear across all tones, how would you determine which ear is better and by how much? A simple test would be to put headphones on the person, play the same level of sound simultaneously in each ear, and determine which side the sound seems to be coming from. Because sound localization is influenced by relative intensity, this method could be used to confirm the existence of a sensory asymmetry. If the sound appears to come from the left, it can be concluded that the right ear (or left hemisphere) is impaired relative to the left ear. To determine the magnitude of the asymmetry, you could then raise the intensity of the sound presented to the relatively impaired ear and/or reduce the intensity of the sound presented to the better ear until the sound seemed to come from neither the left nor right side. The ratio of the sound intensity presented to the impaired ear relative to that of the sound presented to the better ear would quantify the extent of asymmetry. This two-part method is essentially the approach one can take in assessing sensorimotor asymmetries in rats with partial unilateral damage to the brain, and in evaluating treatments.

Behavioral deficits in Parkinson's disease and stroke often can be traced to both sensory and movement initiation problems or an impaired ability to make appropriate motor responses to simple sensory events. In animals, unilateral damage to the sensorimotor cortex, striatum, or nigrostriatal pathway appears to have the perceptual effect of dulling somatosensory and proprioceptive sensory input on one side and, in some cases, enhancing the input from the other side. Asymmetrical sensory deficits, motor reactivity to bilateral sensory input, or predominantly motor dysfunctions can be examined with tests using a two-part method in which an asymmetry is first identified and then the extent of the asymmetry is quantified.

In animal models, it is important to select sensorimotor tests that are sensitive to the brain damage and treatment effects. This chapter describes behavioral tests that have been useful for examining the potential clinical efficacy of interventions that might be beneficial for neurological disorders. It is important to be able to distinguish whether an intervention promotes brain repair mechanisms, saves cells, enhances motor learning and retraining, or reduces the extent of secondary degeneration of tissue. We have chosen to include a subset of sensorimotor tests that we and others have found to be reliable, sensitive, quantitative, and easy to use in rat neurological models. The tests also cover the

range of cellular degeneration typical of focal ischemic injury, nigrostriatal terminal loss, and cervical spinal trauma.

ENVIRONMENTAL ENRICHMENT AND SENSORIMOTOR BEHAVIOR

Most wild rats live in a very complex environment that requires them to navigate obstacles, avoid predators, manipulate objects and circumstances to gain access to food and mates, and so forth, using a wide array of motor skills. By contrast, standard laboratory housing is severely lacking in this sort of stimulation, and laboratory "enriched" environments are still less complex than the rat's natural habitat (Greenough et al., 1976; Jones et al., 2003; Schallert et al., 2003). Even the most sedentary of people do not experience as impoverished an environment as a rat living in an isolated home cage. Therefore, to study sensorimotor behavior in the rat, it may be prudent to make some effort to house animals so that behaviors analogous to natural rat behavior are encouraged.

BILATERAL TACTILE STIMULATION TEST

Rats compulsively groom themselves and respond vigorously to any foreign substance that becomes stuck to some part of their bodies. The adaptive advantages of this behavior may include thermoregulation and maintenance against insects. Somatosensory asymmetries have been effectively determined using a test that involves reacting to, and removing, small sticky stimuli from the forelimbs. It is a two-part test; however, few investigators take advantage of both parts, which are needed to evaluate sensory function independent of the motor component. Practice effects and motor learning play a partial role in the motor aspects of this test but do not affect the sensory side, which can be investigated independently.

SENSORY ASYMMETRY

Small adhesive paper stimuli (Avery adhesive-backed labels, 113 mm^2) are attached to the relatively hairless distal-radial aspect of each of the rat's forelimbs (Schallert et al., 1982, 1983, 2000; Schallert and Whishaw, 1984; Lindner et al., 2003; Fleming et al., 2003) (Fig. 12–1). The rat is placed back into its home cage so that it is not distracted by a novel environment, and it quickly uses its teeth to remove these dots one at a time. In some animals there is a small preoperative bias; in these cases, the hemisphere selected for injury can be opposite to the bias. Also, postoperative outcome can be compared against baseline values for each rat. Rats receiving unilateral lesions to brain areas subserving sensorimotor functions, especially those of the forelimbs, develop an immediate bias for removing adhesive stimuli of similar size from the unimpaired limb first. The order of contacting the ipsilateral versus contralateral stimulus reflects that there is a bias, but the magnitude of the sensory asymmetry requires further evaluation (see later). The latency to remove the stimuli can be used as a measure of motor capacity and is sensitive to practice effects, unlike the order of contact (Schallert and Whishaw, 1984).

Each trial ends when the rat removes both stimuli, or after 2 minutes has elapsed. To avoid habituation to the stimuli, individ-

Figure 12–1. Attaching adhesive stimuli (dots) to a rat's forelimbs in preparation for the bilateral tactile stimulation test.

ual trials should occur at intervals of no less than 5 minutes. In addition, the rats used should be well handled and have received several practice trials with the test before preoperative data are collected. Experience with the test calms the rats and makes the stimuli easier to apply but does not appear to affect actual performance.

This test is generally used to examine sensorimotor integration, although, as indicated earlier, it is possible to some degree to distinguish between the sensory and motor components involved (Schallert et al., 2002). For example, a change in the latency between initial contact and subsequent removal of a dot (i.e., how much time it takes to remove the stimulus) can be an index of sensorimotor function. As in many of the tests presented here, however, it is important that such a change be represented as an asymmetry between the impaired and unimpaired limbs in unilateral lesion models to control for nonmotor and nonsensory factors (e.g., motivational state, alertness) that could have a global influence on latencies to contact and remove the dot. The contralateral (impaired limb) *motor* component of this test is best assessed by determining the time point at which the animal makes contact with the stimulus on the impaired side and scoring how much time *after* that time point it takes to remove that stimulus. This difference then would be compared with a comparable score of intact control animals (i.e., how long after a control rat contacts a given stimulus before it is removed, again controlling for practice effects by equating extent of experience).

MAGNITUDE OF SENSORY ASYMMETRY

The second part of this test is used as a means of measuring the degree of sensory asymmetry. In this part, the size of the dot placed on the impaired limb is progressively increased (by overlapping two dots), while the dot on the unimpaired limb is made smaller (by cutting down one dot). The dot sizes are increased or decreased by 14 mm^2, as illustrated in Figure 12–2, allowing for area ratios ranging from 1.3:1 to 15:1 between the impaired and unimpaired limbs, respectively. A sufficient increase in this ratio leads to a neutralization, and even a *reversal* (with a slightly higher ratio), in the bias for the limb that is contacted first, and the ratio at which this occurs is used as the measure of severity of the sensory asymmetry. This measure is correlated with the amount of brain damage (Schallert et al., 1983; Schallert and Whishaw, 1984; Barth et al., 1990); indeed, a small asymmetry can be detected in rats with simple burr holes in the skull. Animals are started at the 2.2:1 ratio (level 3). If the stimulus is removed from the unimpaired limb first, animals then are tested at *two* levels higher. If the stimulus is removed from the impaired limb first, the animal is tested at *one* level lower. This process is continued until the experimenter has determined between which two levels the bias exists and assigned the rat a score that reflects this ratio (e.g., a score of 2.5 is given if the animal's bias reverses between levels 2 and 3).

Acute and chronic asymmetries on this test have been demonstrated in models of cortical injury and ischemia, parkinsonism, and spinal cord injury (Schallert et al., 2000). As recovery occurs, the ratio defining the magnitude of asymmetry becomes smaller inde-

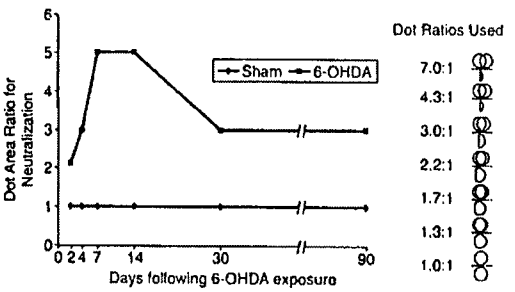


Figure 12–2. (*Left*) Data from an animal model of 6-hydroxydopamine–induced parkinsonism, in which sensory asymmetries measured with the bilateral tactile stimulation test improved over time but did not return to sham-operated control levels. (*Right*) Schematic of the different area ratios of the "dots" used in the test.

pendent of how much or how little practice occurs. Depending on the degree of striatal or nigrostriatal damage, full recovery can occur, even in hemidecorticate rats (Schallert and Whishaw, 1984). However, small changes in the testing environment (e.g., partially opening the home cage while testing) can partially reverse recovery so that the ratio becomes larger, possibly because the striatum is being taxed. This is important because it suggests that the testing environment can have a major influence on measures of functional outcome.

LIMB-USE ASYMMETRY ("CYLINDER") TEST

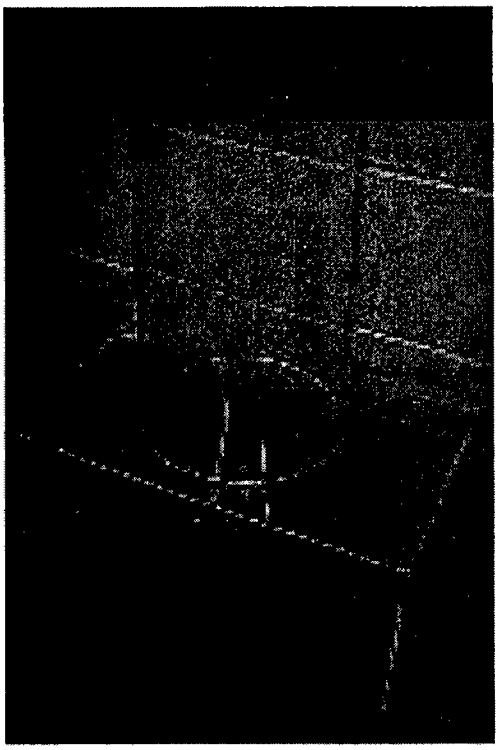
The limb-use asymmetry test evaluates the forelimb use of rats placed in a transparent Plexiglas cylinder. It has been used in a wide variety of motor system injury models, including middle cerebral artery occlusion, spinal cord injury, traumatic brain injury, parkinsonian models, cortical ablation, and focal cortical ischemia (Schallert et al., 2000; Schallert and Tillerson, 2000; Tillerson et al., 2001, 2002; Lindner et al., 2003). A notable feature is a high degree of sensitivity to chronic deficits not noticeably masked by postlesion compensatory behaviors, as well as to chronic sensorimotor deficits that many tests fail to detect. The test is also easy to use and score, has a high interrater reliability, is well correlated with the extent of lesions, including a wide range of dopamine depletion (even 50% or less) (Tillerson et al., 2001), and is relatively unaffected by practice effects or, it seems, the compensatory strategies often adopted by animals after motor system insults (Schallert et al., 2002).

Rats are tireless explorers, in both their natural environments and laboratory home cages. They often explore vertical surfaces by rearing up on their hindlimbs and exploring the surface with their front paws and vibrissae (Gharbawie et al., 2003). The cylinder test takes advantage of this tendency and of the common impairment in the initiation of movement and control of static stable equilibrium, especially center of gravity (Schallert et al., 1979, 1992). A rat is placed in an upright Plexiglas cylinder, open at both ends and measuring 30 cm high by 20 cm in diameter, that rests on a tabletop. The number of independent placements observed for either the right or left forelimb, as well as the number of "both limb" (i.e., simultaneous or near-simultaneous) placements, made onto the inner wall of the cylinder during rears is recorded. These limb placements occur when the rat shifts its weight, touches the cylinder wall, or steps to regain center of gravity during lateral movements along the cylinder wall ("wall stepping").

The data can be recorded over a set period of time in the cylinder or until a certain number of placements has been made. (We prefer the latter technique because different rats, and especially different strains, can vary widely in their activity levels in the cylinder.) To film the rat's behavior for later rating, (*1*) a camera is placed over the cylinder (Fig. 12–3A), (*2*) a camera is positioned to the side of the cylinder with a mirror angled behind and to the side to enable the experimenter to see the rat from all angles during live rating so that no limb movement is missed, or (*3*) the cylinder is placed atop a raised, transparent surface with a mirror positioned beneath at a 45° angle, with the camera aimed at the mirror to film the limb placements from below (Fig. 12–3B). Care should be taken so that the rats do not habituate to the cylinder lest they become inactive. This can be avoided by testing during the dark cycle and by dividing long trials into shorter segments separated by several minutes, during which the rat is placed back in the home cage.

Limb use is scored as the percentage of left, right, or both-limb wall placements relative to the total number of placements observed. One can also obtain a single limb-use asymmetry score by subtracting the percent independent use of the impaired limb from the percent independent use of the

A

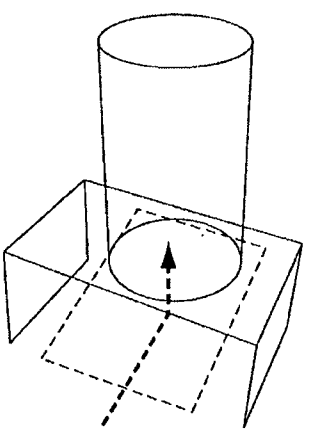


Setup 1:

Place camera directly beneath cylinder.

Setup 2:

Place a mirror at 45° angle beneath cylinder and film at 45° angle to mirror.



B

Figure 12–3. (*A*) Top-down view of a rat making placements in the cylinder, filmed from a camera placed over the cylinder. (*B*) Alternate setup that can be used to film the rat from below the cylinder.

unimpaired limb. Higher numbers indicate a greater bias for use of the unimpaired limb. The former scoring method is advantageous in that it provides more information about both- versus independent-limb use events. It should be noted, however, that even with the latter scoring method, a large number of both-limb use events lower the asymmetry score, albeit not as much as would an equal number of independent impaired-limb placements. An alternative formula is one that we recently adopted because it reduces variability even further and sets a nonbias at 50%:

$$[(\text{ipsi} + {}^1/_2\ \text{both}) \text{ divided by } (\text{ipsi} + \text{contra} + \text{both})] \times 100$$

An additional measure can be obtained from this test. The use of a single limb to make lateral weight-shifting movements, independent of the other limb, is reduced in the impaired limb and enhanced in the nonimpaired limb and reflects a very high degree of functional integrity. That is, when a rat rears and places one forelimb on the wall of the cylinder and then makes a lateral movement to another location on the wall during the same rear sequence, this is considered an independent lateral weight-shifting movement, as opposed to a simple limb placement on the wall (which for the contralateral limb may show some recovery). The number of independent-limb weight-shifting movements along the wall for the ipsilateral forelimb can be compared with that of the contralateral forelimb. After unilateral injury to the sensorimotor cortex, striatum, or other motor areas, such movements are rarely observed in the affected forelimb but are commonly chronic in the unaffected forelimb (where they are even more frequently observed than in either limb of control animals, suggesting a reorganization in the intact hemisphere).

Preoperative baseline values should be obtained before animals undergo surgery or other experimental manipulations. Although there is no consistent population bias in limb preference in the cylinder, some rats do display a predilection for independent use of one limb. When this occurs, experimental lesions can be applied contralateral to this preferred limb so that experimental effects are not confounded by the preexisting limb-use bias. Animals without a preoperative bias can be randomly assigned the lesion side.

Motivation differs between strains of rats. Long-Evans hooded rats, for example, are more active and thus might be considered preferable as animal models, all other considerations being equal. Some rats, especially Sprague-Dawley rats (in our experience), may not initially engage in an adequate amount of wall exploratory behavior in the cylinder. By and large, however, the behavior can be encouraged in any rat with the use of any number of "tricks" that do not affect the limb-use asymmetry score itself, including the following:

- Momentarily turning out the lights in the testing room and testing during red light
- Blowing into or tapping the top of the cylinder
- Placing a dark cage cover (especially the rat's own) over the cylinder
- Placing shavings from the rat's home cage into the cylinder
- Scooting the cylinder (with the rat inside) gently a few cm along the tabletop
- Lightly touching a pencil eraser or cotton tipped applicator to the rat's nose
- Dangling another rat into the cylinder momentarily
- Presenting novel scents or treats at the top of the cylinder
- Picking up the rat and replacing it into the cylinder
- Placing the rat in a new cylinder
- Picking up the rat, flipping over the cylinder, and putting the rat back in

TESTS OF FORELIMB PLACING

Researchers have developed a variety of forelimb-placing tests. Limb placing is usually triggered by visual or vestibular cues or by

contacting the limb being tested with a surface (Wolgin and Kehoe, 1983; Marshall, 1982). Rats use their vibrissae to gain bilateral information about the proximal environment, and this information is integrated between the hemispheres. When the bottoms of all four feet indicate that there is no stable surface for support, the rat is motivated to respond to the first object that one set of vibrissae contacts. In exploring its natural world, the rat frequently encounters surfaces that are unstable or, in the case of a cliff, unsuitable for locomotion. All four limbs must be able to respond to information from either set of vibrissae.

The test we describe next is the vibrissae-elicited forelimb placing test, which uses stimulation of the rat's vibrissae to trigger a placing response (Barth et al., 1990; Schallert et al., 2000; Lindner et al., 2003). This is a nice feature in light of the very important role that the vibrissae play in the rat's sensory environment—indeed, they are thought to be one of the primary tools rats use to explore their world. In addition, the test can be adapted to investigate neural events in the sensorimotor system that occur across the midline (as described next), a feature that is more difficult to implement using other placing triggers.

In this test, the rat's torso is supported by the investigator and suspended such that all four legs hang freely in the air. The experimenter then brings the rat toward the edge of a tabletop or another flat surface, taking care to avoid abrupt movements that might trigger placing due to a vestibular response. If such responses are noted, they should be extinguished by taking the rat through the testing motions in open space (i.e., away from the tabletop) a few times. In the traditional, same-side version of this test, the rat's vibrissae are brushed against the table edge on the same side of the body in which forelimb placing is being evaluated. The percentage of trials in which the rat successfully places its forepaw onto the tabletop is recorded for each side. In addition, the triggering stimulus can be provided by moving the rat head on toward the table edge, thus providing chin-based and/or bilateral vibrissae stimulation, or by holding the rat on its side and stimulating the whiskers *opposite* the limb being evaluated (Fig. 12–4 demonstrates these different types of vibrissae stimulation). In all of these testing scenarios, the experimenter should gently restrain the limb not being tested. Naturally, this requires a tame rat that has been well handled for some time before testing, and which ideally has had a chance to acclimate to the test and the experimenter before being introduced to the experimental manipulation. Trials should be counted only when the rat is relaxed and does not struggle, and achieving this can require a great deal of practice on the part of the experimenter. Intact rats will place with 100% success in all variants of this test.

Figure 12–4. Forms of vibrissae-elicited forelimb placing, demonstrating the proper grip and orientation of the rat for this test.

The same-side version of the test has been used in the evaluation of many central nervous system injury models (Schallert et al., 2000). At our laboratory, we have begun to investigate the recovery of the cross-midline type of placing response in rats receiving cortical (via middle cerebral artery occlusion or focal ischemia to the forelimb area of sensorimotor cortex) or nigrostriatal (via 6-hydroxydopamine infusions to the nigrostriatal bundle) injury. One striking feature noted here is that vibrissae stimulation applied to the "good" (i.e., ipsilesional) side of the body is able to trigger a placing reaction in the impaired forelimb long before stimulation of the contralesional vibrissae can. In contrast, lesions to the nigrostriatal system lead to a complete failure of placing in the contralesional limb in this test, consistent with parkinsonian akinesia. Also, the placing deficit recovers over a period of weeks in the cortical injury models (the rate of recovery depending on the extent of damage to the forelimb area of the sensorimotor cortex and especially to the extent of striatal damage) but persists chronically in parkinsonian models (Felt et al., 2002; Woodlee et al., 2003).

For example, after middle cerebral artery occlusion that damages the striatum, the contralateral forelimb no longer responds to information from the vibrissae about the location of stable surfaces, although the ipsilateral forelimb can respond appropriately to information from the contralateral vibrissae (suggesting that the deficit is not due to a pure sensory impairment associated with the contralateral vibrissae). Moreover, except for severe damage to nigrostriatal dopamine terminals, in which the contralateral forelimb is akinetic, the contralateral forelimb recovers placing in response to ipsilateral vibrissae stimulation. That is, sensory information sent to the intact hemisphere can eventually control motor function associated with the damaged hemisphere, which is typical of normal rats.

TESTS OF HINDLIMB FUNCTION

Rats do not normally use their hindlimbs to initiate or execute complex movement. In this regard, we like to think of rats as being "front-wheel drive," a phenomenon that is illustrated in Figure 12–5, wherein rats supported solely

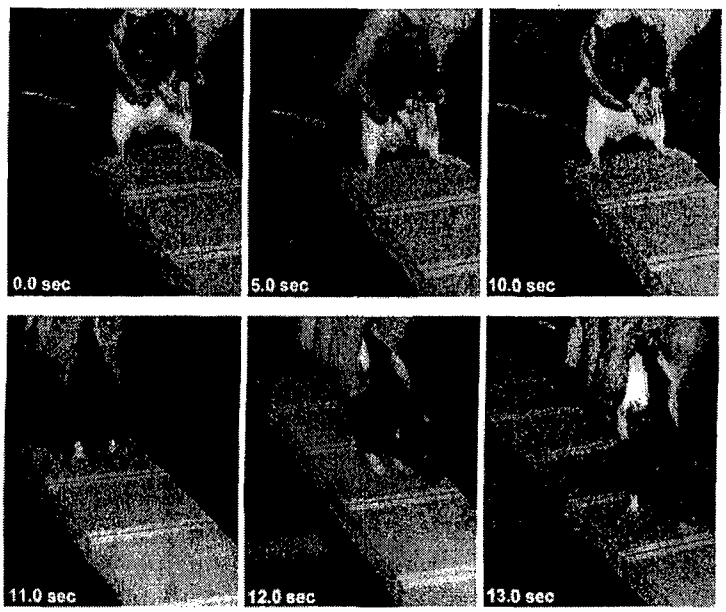


Figure 12–5. Rats operate primarily via "front-wheel drive." This series depicts a rat remaining stationary for 10 seconds when supported only on its hindlimbs (rats do not walk on their hindlimbs even if given more time) but proceeding to move briskly along a beam when flipped over so that it can support its weight on its forelimbs.

on the forelimbs or hindlimbs initiate movement only when on the forelimbs (Schallert and Woodlee, 2003). In part, this is because the vibrissae are not in contact with the ground and thus there is essentially a "stop" signal. This makes testing of hindlimb function rather difficult, but some tests have been developed that provide reliable measures of hindlimb function. This is good news for researchers of spinal cord injury, because experimental animal models of these conditions often involve lesioning caudal to the thoracic cord so that animals can maintain forelimb function and thus continue to care for themselves postoperatively. Also, research into sciatic nerve injury, a widely used model of peripheral nerve damage, can benefit from well-developed tests of hindlimb function.

A relatively new hindlimb test that we developed is the ledged tapered beam–walking test (Schallert et al., 2002). In this test, rats are trained to traverse an elevated beam that is tapered along its extent and has an underhanging ledge (2 cm wide, dropped 2 cm below the upper beam surface) that the rat can use as a crutch if it slips (see Figure 12–6 for dimensions and setup). Footfaults (slips) made with the hindlimbs can be measured as an index of hindlimb function. A footfault can be rated as a half-fault if the paw slips off the upper surface of the beam without falling all the way to the ledge or as a full fault if the paw is placed fully on the ledge. The difficulty of the rat's traverse increases as it moves along the narrowing beam, thus leading to more footfaults. For this reason the beam can be divided into three "bins" of difficulty along its extent, and these can be scored separately or weighted relative to each other to develop a single score. We generally run rats for five trials on a given day of testing, with several minutes between each trial (during which the rat's cagemate, for example, can be tested) to avoid habituation to the test.

An important feature of this test is that the presence of the ledge allows the rat to display a deficit that it might normally make compensatory adjustments to hide. Rats are well known for compensating to overcome lesion-induced deficits, and indeed this can make the development of good behavioral tests difficult. Some compensatory motor adjustments appear to be more automatic in that they appear immediately in response to an impairment, whereas other adjustments require new learning. If one wishes to test the direct effect of a therapeutic intervention on the system in question, it is important to have tests that will target the deficit directly and be minimally affected by these compensatory be-

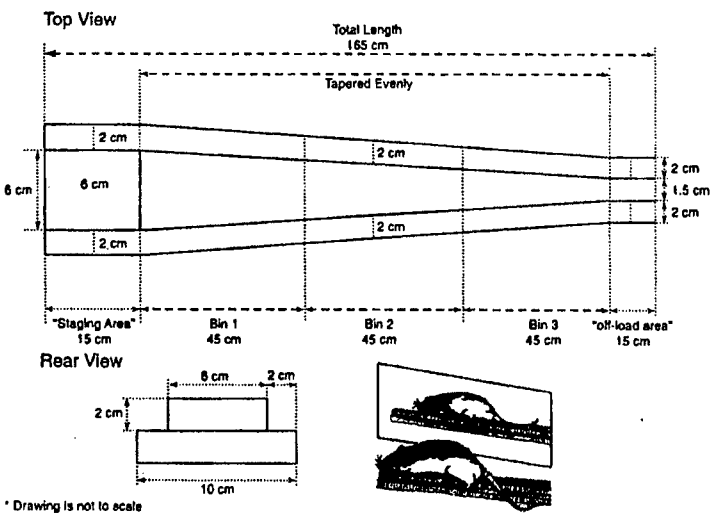


Figure 12–6. The ledged tapered beam, showing dimensions for construction. (*Lower right*) Rat running the beam, making a full left hindlimb footfault (slip) onto the ledge.

haviors. If the test is influenced by compensation, it may not be clear if the therapy is actually ameliorating the deficit per se rather than enhancing motor learning mechanisms that allow for development of the compensatory behavior. Beam-walking tasks that do not use a ledge are plagued by this problem, because rats very quickly learn to make compensatory postural adjustments to keep themselves from falling off the beam. Limb dysfunction may still exist, but the shift in body weight can hide it. With the ledged beam, there is less threat of falling and therefore compensation is less of a problem. In fact, a beam with a detachable ledge can be used to measure the ability of the rat to learn compensatory skills. For example, even several weeks after the insult, rats sustaining brain damage due to middle cerebral artery occlusion (a commonly used stroke model) continue to show a stable deficit on the ledged beam test (Schallert et al., 2002). If the ledge is removed, however, the rats learn to compensate over the course of just a few trials until they are running the beam successfully with no footfaults. This does not necessarily indicate recovery of limb function, though, because rats will begin to make foot faults again if the ledge is subsequently replaced. The speed with which rats are able to shift between displaying a deficit on the ledged beam and learning to compensate in the ledge's absence may be reflective of the level of impairment and the capacity of motor learning circuits that may or may not have been affected by the lesion.

Some hints make the use of the beam more successful. Preoperatively, rats must be trained to run the beam without fault, and preferably without stopping to explore the beam or its surroundings during the run. There is no prescribed number of training trials needed to achieve this result; each rat can simply be trained until this criterion is reached. Good training eases the testing phase, because stopping to encourage the rat to traverse the beam becomes less necessary. When setting up the beam, the experimenter may want to place the rat's home cage at the end to serve as a reinforcer. The cage may also be covered with a dark cloth to make it more enticing. During the initial training, the experimenter can encourage the rat to run by tapping on the beam in front of the rat, picking up the rat's tail from behind to encourage it to move away, or "tucking" the rat's hindquarters with the experimenter's hands. On early trials, the rat frequently stops to sniff the beam or have a look around the testing room, but this generally ceases during the course of pretraining. Objects should not be placed to the side or below the beam because these tend to distract the animal. A comprehensive review of the setup, use, and scoring of the beam can be found in Schallert et al. (2002).

Other opportunities exist for testing hindlimb function. Although rats rely primarily on their forelimbs for most movement, the hindlimbs are used in behaviors such as jumping, swimming (in which the forelimbs usually stay stationary as the hindlimbs paddle [Whishaw et al., 1981; Kolb and Tomie, 1988; Stoltz et al., 1999]), and backing out of tunnels or other tight areas in which the rat cannot turn around. They can also use the hindlimbs to maintain balance during a rear; one can also quantify hindlimb stepping in the cylinder test (see earlier) as an index of hindlimb function, if the cylinder is set up to be filmed from below (Fleming et al., 2002). In the cylinder, rats with unilateral nigrostriatal system damage mimicking a hemiparkinsonian state tend to leave the impaired hindlimb planted in one place and pivot around this akinetic limb by stepping with the unimpaired limb.

CONCLUSION

The sensorimotor tests described earlier are certainly not the only ones that should be considered useful, but in our experience these

qualify as among the best for assessing functional outcome after unilateral focal ischemic injury, nigrostriatal degeneration, traumatic head injury, damage to the intrinsic neurons of striatum, and cervical spinal hemisection. It is possible to use aspects of these tests along with others to determine the location and extent of injury and the degree of improvement over time. With practice, investigators can reliably and rapidly evaluate treatment effects. Our Web site (http://www.schallertlab.org) has downloadable videos and information that can help new researchers adopt these and related tests of sensory and motor function.

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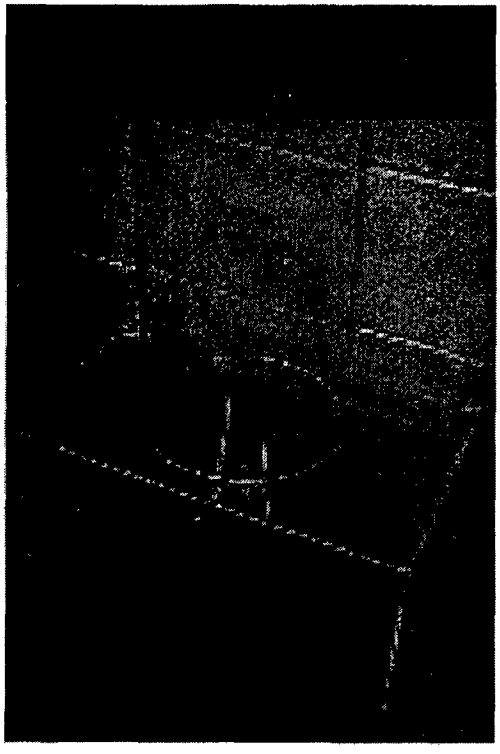

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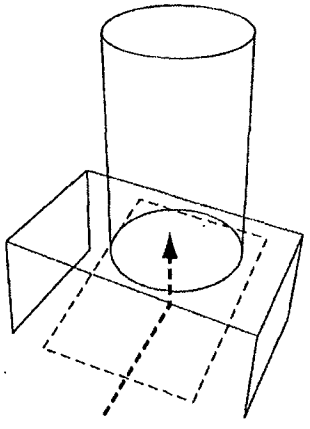

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Figure 12–3. (*A*) Top-down view of a rat making placements in the cylinder, filmed from a camera placed over the cylinder. (*B*) Alternate setup that can be used to film the rat from below the cylinder.

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contacting the limb being tested with a surface (Wolgin and Kehoe, 1983; Marshall, 1982). Rats use their vibrissae to gain bilateral information about the proximal environment, and this information is integrated between the hemispheres. When the bottoms of all four feet indicate that there is no stable surface for support, the rat is motivated to respond to the first object that one set of vibrissae contacts. In exploring its natural world, the rat frequently encounters surfaces that are unstable or, in the case of a cliff, unsuitable for locomotion. All four limbs must be able to respond to information from either set of vibrissae.

The test we describe next is the vibrissae-elicited forelimb placing test, which uses stimulation of the rat's vibrissae to trigger a placing response (Barth et al., 1990; Schallert et al., 2000; Lindner et al., 2003). This is a nice feature in light of the very important role that the vibrissae play in the rat's sensory environment—indeed, they are thought to be one of the primary tools rats use to explore their world. In addition, the test can be adapted to investigate neural events in the sensorimotor system that occur across the midline (as described next), a feature that is more difficult to implement using other placing triggers.

In this test, the rat's torso is supported by the investigator and suspended such that all four legs hang freely in the air. The experimenter then brings the rat toward the edge of a tabletop or another flat surface, taking care to avoid abrupt movements that might trigger placing due to a vestibular response. If such responses are noted, they should be extinguished by taking the rat through the testing motions in open space (i.e., away from the tabletop) a few times. In the traditional, same-side version of this test, the rat's vibrissae are brushed against the table edge on the same side of the body in which forelimb placing is being evaluated. The percentage of trials in which the rat successfully places its forepaw onto the tabletop is recorded for each side. In addition, the triggering stimulus can be provided by moving the rat head on toward the table edge, thus providing chin-based and/or bilateral vibrissae stimulation, or by holding the rat on its side and stimulating the whiskers *opposite* the limb being evaluated (Fig. 12–4 demonstrates these different types of vibrissae stimulation). In all of these testing scenarios, the experimenter should gently restrain the limb not being tested. Naturally, this requires a tame rat that has been well handled for some time before testing, and which ideally has had a chance to acclimate to the test and the experimenter before being introduced to the experimental manipulation. Trials should be counted only when the rat is relaxed and does not struggle, and achieving this can require a great deal of practice on the part of the experimenter. Intact rats will place with 100% success in all variants of this test.

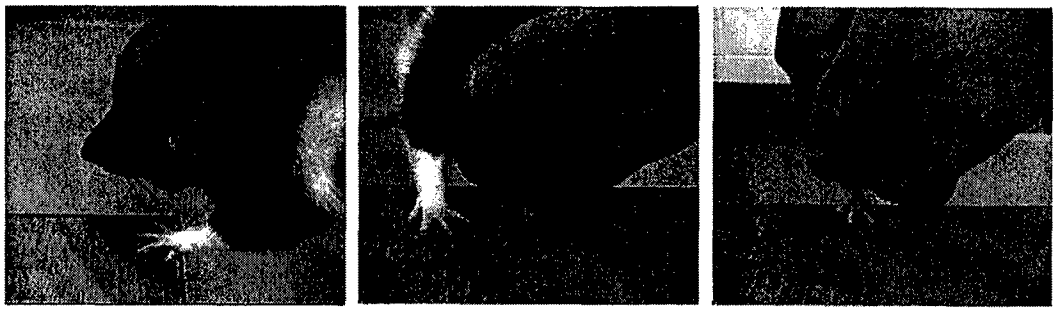

Figure 12–4. Forms of vibrissae-elicited forelimb placing, demonstrating the proper grip and orientation of the rat for this test.

A behavioral marker of non-dopaminergic damage in the 6-OHDA model of Parkinson's disease

Woodlee MT, Kane JR, Schallert T

Rats sustaining unilateral nigrostriatal dopamine depletion caused by microinfusion of 6-hydroxydopamine (6-OHDA) show a variety of sensorimotor deficits, including a dramatically reduced ability to place the contralateral forelimb onto a supporting surface in response to vibrissae sensory stimulation or to vibrissae plus forelimb contact. Here we show that this forelimb placing deficit, unlike many other parkinsonian behaviors elicited by 6-OHDA, was not reproduced in intact animals by robust acute blockade of dopamine receptors with either SCH-23390 (a DA1 subfamily receptor antagonist), haloperidol (a DA2 subfamily antagonist), or a combination of the two drugs. We tested the effects of SCH-23390, haloperidol, and reserpine, as well as the effects of treatment by L-DOPA or apomorphine, in intact vs. unilaterally or bilaterally 6-OHDA-lesioned rats. Interfering with DA synaptic activity by drugs alone did not block forelimb placing in intact animals. Nor did it block placing of the ipsilateral forelimb after unilateral 6-OHDA lesions or restore placing of the contralateral forelimb (which rules out cross-hemispheric inhibition as a factor). The ability of DA agonists to restore placing in lesioned animals appeared to depend on the synaptic site of action of the drugs. The evidence suggests that in 6-OHDA-lesioned animals, the placing deficit may be due to DA terminal loss together with nonspecific damage outside of the nigrostriatal dopamine pathway. Placing capacity may be a sensitive index of neurotoxin specificity to nigrostriatal neurons. The findings may have implications for modeling idiopathic Parkinson's disease, in which non-dopaminergic degeneration is gaining recognition as an important component of the disease's pathology.

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Abstract Title: A Methodology for In Vivo Detection of the Effects of Dopamine Transients on Glutamate Evoked Striatal Activity Using Electrophysiological, Fast-Scan Cyclic Voltammetric and Micro-Iontophoretic Techniques

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Abstract: Transient changes in dopamine (DA) levels or phasic DA signaling has been implicated in social interaction, and drug and food seeking. Electrically evoked DA transients have been shown to regulate single unit activity of spontaneously active striatal neurons in a biphasic manner, causing excitation at low DA levels and inhibition at high DA levels. However, surprisingly little is known about what effect DA transients have on glutamate (GLU)-evoked single unit activity in the striatum. We have combined the techniques of extracellular electrophysiology with micro-iontophoresis (IONT) and fast-scan cyclic voltammetry (FSCV) at a carbon-fiber microelectrode (CFM) to investigate the relationship between DA transients and GLU evoked single unit activity of MSN.

Piggy-back electrodes were made with CFM and a 5 barrel micropipette for IONT. This electrode configuration posits many advantages in terms of drug delivery, ejection current interference and detection capability compared to techniques used previously. No significant difference in peak to peak amplitudes of action potentials was found between single-barrel glass electrodes and CFM. However, CFM yielded a significantly lower noise level. In addition, FSCV can characterize and optimize iontophoretically applied DA to mimic physiological DA transients both in vitro and in vivo. Furthermore, preliminary data demonstrates an inhibition of GLU-evoked striatal unit activity by high concentrations of iontophoretically applied DA.

Because voltammetrically detected DA transients evoked at physiological stimulation frequencies decrease proportionally to the degree of denervation of the nigrostriatal pathway, we intend to employ this technique to characterize the effects of DA transients on single unit activity in the striatum in an animal model of preclinical Parkinson's disease.

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APPENDIX 5

The Journal of Neuroscience

Simultaneous measurements of dopamine release and nucleus accumbens cell firing at the same probe reveal different temporal scales of signal encoding in awake rats

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Simultaneous measurements of dopamine release and nucleus accumbens cell firing at the same probe reveal different temporal scales of signal encoding in awake rats

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Keywords: dopamine, nucleus accumbens, unit firing, cyclic voltammetry

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Abstrac
t

While dopamine is involved in motivated behaviors, the effects of its transient release on
postsynaptic neuronal activity are unclear. We combined electrophysiology and voltammetry at
the same electrode in awake, unrestrained rats to show that electrical stimulation of the medial
forebrain bundle (MFB) that elicited dopamine release in the nucleus accumbens (NAc)
simultaneously produced time-locked changes (inhibitions and/or excitations) in the activity of a
subset of NAc neurons. The excitations were mainly antidromic and thus directly activated.
However, the inhibitions in firing rate appeared to be modulated by $GABA_A$ rather than
dopamine receptors. Nevertheless, repeated MFB electrical stimulation elicited long-term
changes in the baseline firing rate of NAc units that were altered by blocking vesicular dopamine
release. These results show that noncontingent MFB stimulation comprises a rapid encoding
component in the NAc mediated by GABA and a slow modulatory signal conveyed by
dopami
ne.

In recent years, the NAc has been under intense investigation due to its role in the mesolimbic reward circuit in the brain (Self and Nestler, 1998; Bassareo and Di Chiara, 1999; Horvitz, 2000). This structure is critical for integrating information related to reinforcement as it contributes to behavioral selection in response to predictive cues, sequencing of behaviors, and learning (Koob and Bloom, 1988; White, 1990; Robbins and Everitt, 1996). One of the critical neurotransmitters involved in this reward circuitry is dopamine, whose neurons arise from the ventral tegmental area (VTA) and project through the MFB to the NAc (Ikemoto and Panksepp, 1999). Electrophysiological recordings in the primate VTA have revealed that dopaminergic neurons respond to rewards with a transient burst in activity (Montague et al., 1996; Schultz et al., 1997; Berridge and Robinson, 1998; McClure et al., 2003). When the animal is well trained, cues that predict reward also result in bursts. Such extensive characterization has not been performed in rodents; however, existing data indicates similar responses of dopaminergic neurons to unexpected or rewarding stimuli (Hyland et al., 2002).

While recording terminal dopamine release with fast-scan cyclic voltammetry (FSCV) in behaving rats, we have observed transient concentration surges of dopamine. These can serve as conditioned signals during cocaine seeking (Phillips et al., 2003; Stuber et al., 2005) and also occur when the animal is alerted (Robinson et al., 2002) or exposed to natural reinforcers (Roitman et al., 2004). These transients typically last less than 2 s, are time-locked to behavioral cues, and achieve mean concentrations above 70 nM (Phillips et al., 2003). These characteristics are exactly those anticipated to arise from phasic dopaminergic cell firing. Furthermore, we have shown that dopamine transients, unlinked to any apparent stimuli, can be generated following acute administration of different drugs (Cheer et al., 2004; Robinson and Wightman, 2004).

Reward circuitry in the brain should allow rapid processing of information because

motivated behaviors, essential for survival, often take place within seconds. Indeed, single-unit electrophysiological recordings have shown that NAc neurons rapidly encode information related to actions underlying motivated behavior (Carelli and Wightman, 2004; Yun et al., 2004; Deadwyler et al., 2004; Mulder et al., 2005). Dopamine, however, is viewed as a slow acting neurotransmitter that operates through G-protein coupled receptors whose transduction requires time to activate the intracellular processing that leads to changes in neuronal excitability (Benians et al., 2003; Girault and Greengard, 2004). Thus, despite the rapid response of dopaminergic neurons to reward-based stimuli and the accompanying rapid dopamine release, the physiological consequence of fast dopamine signaling remains unclear.

To begin to address this issue, we have combined electrophysiology and FSCV at the same carbon-fiber electrode in awake, unrestrained rats to allow simultaneous monitoring of dopamine transients and neuronal activity at the same location within the NAc. The combined approach was pioneered by Millar and coworkers (Armstrong-James et al., 1980), but has never been used in unanesthetized animals. For this initial study, dopamine transients were directly evoked by the experimenter with electrical stimulation of the MFB through which dopaminergic neurons project rostrally to the NAc. The results provide a possible interpretation of the role that fast dopaminergic signaling exerts on NAc neuronal activity.

Materials and Methods

Animals and surgery

All animals were treated in accordance with the regulations established by the Guide for the Care and Use of Laboratory Animals (NIH). Male Sprague-Dawley rats implanted with jugular vein catheters (Charles River, Wilmington, MA) weighing between 300 and 350 grams were used as

subjects. Rats were single-housed in cages with ad libitum access to food and water within temperature-regulated rooms (22-23 °C) with artificial lighting provided on a 0700-1900 hrs cycle and experiments performed during the light cycle. Surgeries were carried out as described previously (Cheer et al., 2004). Briefly, a guide cannula cut to 2.5 mm (Bioanalytical Systems, West Lafayette, IN) was positioned above the NAc core (+1.3 mm AP, +1.3 mm ML, relative to bregma). An Ag/AgCl reference electrode was placed in the contralateral hemisphere. All items were affixed to the skull with machine screws and cranioplastic cement. A detachable microdrive containing a cylindrical carbon-fiber microelectrode (75-100 µm length of exposed fiber, 6 µm in diameter, T-650; Amoco, Greenville, SC) was inserted into the guide cannula and the electrode was lowered into the dorsal NAc core. A bipolar stimulating electrode placed directly above the MFB (-4.6 mm AP, +1.4 mm ML, and -7.7 to -8.8 mm DV) was lowered in 0.2 mm increments until electrically-evoked (60 biphasic pulses, 60 Hz, 125 KA, 2 ms/phase) dopamine release was detected. The stimulating electrode was cemented and the carbon-fiber electrode was removed and replaced with a stylet until the day of the experiment.

Data acquisition

Electrophysiology

A carbon-fiber electrode was lowered into the NAc core, the electrode was locked in place, and the carbon-fiber and Ag/AgCl electrodes were connected to encapsulated head-mounted operational amplifiers via gold connectors. Signals were routed to an electrical swivel (Med-Associates, St Albans, VT) located at the top of the test chamber which allowed animals to move freely during recording sessions. Action potentials recorded extracellularly from carbon fiber electrodes (impedance at the tip: ~500 kM at 1 KHz) were amplified (fixed gain: x1000) and bandpass-filtered (300 Hz to 3 KHz) on custom-built instrumentation (Chemistry Department

Instruments Shop, UNC Chapel Hill, Chapel Hill, NC) and digitized using commercially available software (Digitizer, Plexon Inc, Dallas, TX). Time-amplitude window isolated spikes were sorted and counted separately as a function of waveform offline (Offline Sorter, Plexon Inc, Dallas, TX). Typically, one to two neurons were observed at any given location. Custom-written software (LabVIEW, National Instruments, Austin, TX) was used to monitor behavioral timestamps in the electrochemical record, whereas electrophysiological and behavioral timestamps were integrated into a master file for offline analyses using Neuroexplorer (Plexon Inc, Dallas, TX). Isolated neurons that met inclusion criteria as determined by: amplitude-over-noise band (3:1), overall firing rate (1 - 10 Hz), waveform (50 - 180 KV), noise-free autocorrelograms, inter-spike intervals with a clearly defined refractory period, power spectra with no extraneous (i.e., 60 Hz) oscillations and valid PEHs.

Combined electrophysiological and electrochemical measurements

FSCV measurements were made every 200 ms by applying a triangular waveform (-0.6 V to +1.4 V, 400V/s). Data was stored to a PC running custom-written LabVIEW software (National Instruments, Austin, TX). To stabilize the electrochemical response, the voltammetric waveform was applied for 15 min at a frequency of 60 Hz. Dopamine release was then optimized within the NAc core by adjusting the dorsoventral (D/V) position of the working electrode in 0.3 mm increments. Once the acquisition of low-noise extracellular single-unit recording of neurons was achieved in a location where dopamine release could be measured voltammetrically, a solid-state relay in the headstage assembly was engaged to electronically alternate from a current amplifier to a voltage follower between voltammetric scans. Triangular voltammetric input waveforms (- 0.6 to +1.4 to -0.6V) were alternated with electrophysiology (voltage) acquisition so that spike records showed gaps at the frequency at which the voltammetric scan was applied. Pilot work

showed that the optimal frequency for application of the voltammetric waveform was 5 Hz. Thus, voltammetric scans lasted for 20 ms, and the remaining 180 ms were used to collect extracellular single-unit recording. Both signals were referenced to the Ag/AgCl electrode that was routed to ground.

Neither measurement affected the other. A raw spike record obtained after amplification and filtering for a single voltammetric scan along with a circuit diagram for the switched headstage (see Supplementary Figure online). It shows that neuronal spikes can be readily obtained once the relay allows for the collection of voltage readings. Since carbon fiber electrodes were approximately 100 Km long, collection of multiple single-unit activity occurred frequently and was sorted offline using principal component analysis. Following collection of data at one D/V coordinate, the electrode was moved ventrally at least 150 Km (to prevent collection of the electrical field from the preceding unit) until another neuron and release location were found.

Data analysis

Cyclic voltammograms (used for identification of detected species) were background subtracted from 10 cyclic voltammograms obtained before the stimulation. Two-dimensional plots with time as the abscissa, voltage as the ordinate, and the background subtracted current encoded in false color were utilized to identify the detected species. Dopamine changes were resolved by plotting the current at the peak oxidation of dopamine (+ 0.6 V vs. Ag/AgCl) as a function of time. Data were pH-corrected as described previously in order to obtain dopamine-associated currents that were free of non-specific ionic contributions.

Electrophysiological data analysis consisted of assessment of single session strip charts and composite PEHs constructed around the stimulation. The time window of analysis PEHs was ± 15 s around the stimulation during noncontingent stimulations and ± 5 s for pharmacological

studies on E-type cells. Each PEH was partitioned into three different epochs to allow comparison of the temporal structure of changes in firing related to the stimulation. Pre- and post-response intervals were used as baseline/recovery firing rates, and a prescribed interval around a time-locked peak/trough of firing in the PEH (response) was chosen for analysis as follows: noncontingent stimulations (baseline: -15 to 0 s; response: 0 to +5 s; recovery: +5 to +15 s) and E-type cells for pharmacology (baseline: -5 to 0 s; response: 0 to +3 s; recovery: +3 to +5 s). Two-min bins were used for firing rate comparisons within stripcharts. Perievent histograms were divided into 6 equal epochs for signal-to-baseline analysis and the fourth (response) epoch (0 to +5 s for inhibitions; 0 to + 0.8 s for excitations) was divided by the first (baseline) epoch (-15 to -10 s for inhibitions; -5 to -4.2 s for excitations)

Statistics

Time-stamped I vs. t plots and data from individual cell PEHs was analyzed with ANOVA and Newman-Keuls post-hoc tests. Conventional t tests for dependent (within cell type comparisons) or independent (between cell type comparisons) measures were utilized to determine statistical differences in peak dopamine release, firing rates and signal-to-noise ratios (p Q 0.05). For duration analyses, baseline was arbitrarily defined as the return to noise levels where uptake was greatest. The onset of recovery of firing following the time-locked period from composite PEHs was defined as the time bin where changes in firing rate were no longer significantly different from baseline.

Drugs

SCH23390 (RBI, Natick, MA), raclopride (Sigma-Aldrich, St Louis, MO, USA) and bicuculline (Sigma-Aldrich, St Louis, MO) were freshly suspended in heparinized saline (0.9%) for intravenous injection. RO4-1284 was a generous gift from Roche Pharmaceuticals (Palo Alto,

CA) and was also dissolved in saline.

Histology

Upon completion of the study, selected animals were anesthetized with a lethal dose of sodium
urethane (2 g kg^{-1}, i.p.). A constant current of 20 KA was applied for 20 s through the carbon-fiber at locations were recordings had been made to generate electrolytic lesions for later visualization under bright field microscopy. Animals were then transcardially perfused with 300 ml of saline followed by 300 ml of a 10% formalin solution. Brains were removed, cryoprotected and coronally sectioned at 40 µm on a cryostat.

Results

Dopamine release and neuronal firing patterns in the NAc during MFB electrical stimulation

Simultaneous measurements of dopamine release and single-unit activity were made with a carbon-fiber electrode in the NAc while rats were stimulated through an electrode chronically implanted in the MFB (24 biphasic pulses, 60 Hz, 125 KA, 2 ms per phase in the case of Figure. 1A). Each stimulus train resulted in a robust increase in extracellular dopamine concentration that was stable across the behavioral session (Figure 1A). The color representation of the voltammetric current below the concentration versus time trace shows the oxidation (green circle) and the reduction (black circle) of dopamine. A background subtracted cyclic voltammogram obtained at the oxidation potential for dopamine and at the peak release elicited by the stimulus (dashed lines) is shown to the right of the color plot. The perievent raster and associated histogram centered on the electrical stimulation (green dashed line at time 0) shown in Figure 1B, displays the activity of the unit (waveform to the right) that was simultaneously recorded at the site where the data shown in Figure 1A was collected. The firing rate of this unit was consistently inhibited for the 10 trials shown, and the overlaid trace (red) shows the averaged evoked dopamine release signal, which did not decay during the same number of trials.

Similar experiments were performed at the same carbon-fiber electrode where dopamine release and single-unit activity were monitored in the NAc. One of two different stimulus trains was evaluated in individual animals (n = 13). The "long" stimulation (24 biphasic pulses, 60 Hz, 125 KA, 2 ms per phase) was identical to that used during previous self-stimulation experiments in our laboratory (Garris et al., 1999). We also evaluated responses to "short" stimulations (6 biphasic pulses, 30 Hz, 125 KA, 2 ms per phase) that mimic the bursts that dopaminergic neurons periodically exhibit in freely moving rats (Hyland et al., 2002).

A total of 162 NAc neurons were recorded for both types of noncontingent stimulations. Of 162 cells, 112 (69%) exhibited one of three types of patterned discharges relative to stimulus onset, and the remaining 50 neurons (31%) were unaffected. Composite perievent histograms (PEHs) displaying the average firing rates of each cell type relative to the onset of the long electrical stimulation train demonstrates the different responses obtained (Figure 2). As noted above, some NAc neurons (n = 24) were unaffected (termed U-type) by the robust dopamine changes measured simultaneously (Figure 2, Table 1; $F_{(2,71)} = 0.04$; $p = 0.9$). In contrast, another subset of NAc cells were significantly inhibited by the stimulation train (n = 46, termed I-type; Figure 2, Table 1; $F_{(2,125)} = 3.83$; p Q 0.05). The average duration of the inhibition in cell firing of I-type neurons (5.3 ± 0.9 s, Figure 2) closely matched that of evoked changes in dopamine concentration (4.2 ± 0.2 s). Finally another population of neurons (n = 12) showed a mixed response with inhibitory followed by excitatory components; this category was termed inhibited/excited (I/E-type). I/E-type neurons displayed a modest inhibition compared to I-type neurons that was followed by a "rebound" excitation (Figure 2, Table 1; $_{(2,35)} = 2.2$; p Q 0. 05).
F

When responses to the short stimulation were assessed, U-type (n = 26) and I-type (n = 40) cells were also encountered (Figure 3A). Across all U-type cells, firing rate was not significantly modified by the stimulation ($F_{(2,77)} = 0.01$; $p = 0.7$). In contrast, I-type neurons displayed a significant inhibition in firing rate relative to stimulation onset (Figure 3A, Table 1; $F_{(2,125)} = 3.83$; p Q 0.05). The duration of the inhibition for I-type neurons (Figure 3A; 4.4 ± 1.2 s) tightly followed the evoked changes in dopamine concentration. Interestingly, I/E responses were not observed with the short stimulation train; however, some neurons (n=14) showed exclusive excitatory profiles (termed E-type; Figure 3A, Table 1; $F_{(2,41)} = 2.5$; p Q 0.05). The duration of this stimulation-induced excitation (3.8 ± 0.8 s) matched the duration of evoked

dopamine release (3.4 ± 0.2 s; Figure 2A). However, a large proportion of E-type units (~67 %) were antidromically driven by the short electrical stimulation as evidenced by their mean latency time to fire (13.9 ± 4 ms) after each stimulus pulse (Wolske et al., 1993) (Figure 3A$_1$).

As noted above, the short stimulation train was chosen to enable the detection of dopamine release events that resemble burst activity of dopaminergic neurons (6 pulses, 30 Hz). Indeed, the color plot in Figure 3B shows not only a stimulation-evoked dopamine release event but the voltammetric current and its color representation also show a non-stimulated dopamine transient$_{16}$ (asterisk) that occurred seconds before application of the short stimulation (black bar). The cyclic voltammograms (obtained at the potential and times indicated by the dashed lines) for the two release events are shown to the right (Figure 3B $_1$). The amplitude of the naturally occurring transient was similar to that elicited by the stimulation (black bar) and the shape of its voltammogram correlated well with the evoked signal ($r_2 = 0.75$), indicating that the change in current was indeed due to dopamine oxidation. This confirms that the electrochemical technique used here (Heien et al., 2003) is adequate for the detection of physiologically relevant dopamine release events.

The maximal dopamine concentration was significantly higher with the long stimulation (red trace in Figure 2) compared to the short stimulation (red trace in Figure 3A; 530 ± 63 nM and 115 ± 20 nM, respectively; t = 9.6; p Q 0.01, note different dopamine concentration scales). However, the amplitude of electrically-evoked dopamine release was not significantly different at locations where the different categories of cells (U, I, E, or I/E) were recorded following the long or the short stimulation ($F_{(2,243)} = 1.73$, $p = 0.18$ and $F_{(2,237)} = 2.33$, $p = 0.1$; respectively). Interestingly, peak firing rate for E-type cells was significantly higher than the rebound excitation elicited by I/E cells (Table 1; t = -2.1; p Q 0.05), even if dopamine release was ~60 %

less at locations where E-type cells were observed (E-type responses were only recorded following the short stimulation).

Effect of dopamine receptor antagonists on NAc neuronal responses time-locked to noncontingent MFB electrical stimulation

The above results show that noncontingent electrical stimulation of the MFB evokes changes in NAc neuronal firing that coincide with phasic dopamine concentration changes. Here, we tested whether time-locked responses observed following noncontingent stimulation may be mediated by dopamine receptors by repeating the experiments in a subset of neurons in the presence of specific dopamine receptor antagonists. To approximate the involvement of dopamine receptors in the regulation of naturally occurring cell firing (i.e., bursting), the short stimulation was used in these experiments.

Blockade of D1-like receptors by SCH23390 (SCH) administration (40 Kg kg-1, i.v.; Shi et al., 1997) did not alter I-type (p Q 0.2) or E-type (p Q 0.8) firing patterns (Figure 4A). Similarly, D2-like receptor blockade with raclopride (RACLO; 80 Kg kg-1, i.v.; Shi et al., 1997) did not modify the time-locked response of I-type (p = 0. 07) or E-type (p = 0. 09) cells (Figure 4B). Nonetheless, RACLO significantly elevated evoked maximal dopamine recorded near I-type and E-type cells (from 152 ± 31 nM to 243 ± 64 nM; t = -4.4; p Q 0. 0001 and from 118 ± 21 nM to 212 ± 57 nM; t = -3.6; p Q 0. 0001, respectively, Figures 4A and 4B), consistent with its known actions on dopamine release (Gonon and Buda, 1985). This enhancement was evident ~ 90s post-injection. Thus, the dose used was efficacious at D2-like receptors in the NAc. The antidromic nature of the evoked change in firing of a subset of E-type neurons (Figure 3A) may explain the lack of effect of dopamine receptor antagonists on this population. Because dopamine is known to alter background firing rates of striatal output neurons (Kiyatkin and Rebec, 1999)

we next examined whether SCH or RACLO altered the signal-to-baseline ratio of time-locked inhibitions and excitations. Neither D1-like dopamine receptor blockade nor D2-like receptor blockade modified signal-to-baseline ratios for I-type ($p = 0.83$ and $p = 0.64$, respectively) or E-type ($p = 0.41$ and $p = 0.48$, respectively) responses.

We further probed dopaminergic effects on I-type time-locked responses with the administration of RO4-1284 (RO; 1 mg kg^{-1}, i.v.), an inhibitor of the vesicular monoamine transporter ($n = 7$). I-type units were selected because they form the largest category of phasic neurons and are devoid of antidromicity confounds. This drug reduced stimulated dopamine release from 243 ± 91 nM to undetectable levels (Figure 5). As seen in the composite PEHs of Figure 5, the stimulation-induced inhibition of firing persisted (pre-RO firing rate at trough: 2.03 ± 0.8 Hz vs. post-RO: 1.81 ± 0.7; $t = -1.6$; p Q 0.08), although baseline firing rate significantly decreased (pre-RO baseline firing rate: 4.67 ± 1.1 Hz vs. post-RO: 2.78 ± 0.9 Hz, $t = 20.6$; p Q 0. 0001). This inhibition in baseline activity was accompanied by a significant decrease in the signal-to-baseline ratio of the inhibitory response following RO ($t = -7.2$; p Q 0. 0001).

Effects of bicuculline, a $GABA_A$ receptor antagonist, on NAc neuronal inhibitions time-locked to noncontingent MFB electrical stimulation

We next examined whether I-type responses were mediated by the predominant inhibitory brain neurotransmitter, GABA. GABAergic neurons project through the MFB to the NAc (Van Bockstaele and Pickel, 1995) and thus could also be depolarized by the electrical stimulation. The $GABA_A$ receptor antagonist bicuculline (BIC) was administered in the presence of RO at a non-epileptogenic dose (2 mg kg^{-1}, i.v.). Blockade of $GABA_A$ receptors further decreased baseline firing rate ($t = 13.4$; p Q 0.0001, Figure 5). In addition, I-type responses modified their patterned discharge to an E-type response (from 0.56 ± 0.2 Hz at baseline to 1.31

± 0.2 Hz at the peak; t = -3.75; p Q 0. 001) while evoked dopamine changes remained undetectable. These excitatory responses were not time-locked to each stimulus pulse within the train and latencies varied greatly from one pulse to the next (128 ± 36 ms; range 38-325 ms) consistent with synaptic, rather than antidromic, activation.

Noncontigent MFB electrical stimulation produces slow changes in accumbal cell firing

In addition to our examination of NAc cell firing relative to MFB stimulation on a short timescale (seconds), we also investigated accumbal firing patterns for the same population of neurons described above over longer time intervals (minutes). Representative stripcharts from three different NAc neurons (waveforms to the right) recorded over entire non-contingent intracranial stimulation sessions are shown in Figure 6. The records show the discharge characteristics of each cell at 5 s resolution and associated stimulations occurring at 1 min intervals in the session (tick marks on top). Neurons were partitioned into three populations based on their long-term changes in firing related to MFB stimulation. The main population showed no change in mean firing rate as a result of noncontingent stimulation (top; n = 39). This subset of cells were previously classified on the short time scale as U-type (n = 13 cells), I-type (n = 21 cells) and I/E-type (n = 5 cells). However, there were neurons that gradually increased (middle; n = 32; short timescale: 7 U-type, 20 I-type and 5 I/E-type) or decreased (bottom; n = 11; short timescale: 4 U-type, 5 I-type and 2 I/E-type) their mean overall firing rates as the number of stimulations increased. Thus, analysis on the longer timescale revealed that neuronal activity was progressively modulated for a population of NAc cells by entire sets of noncontingent stimulations on a minute-to-minute time scale. However, this slow modulation was not a good predictor of a given type of phasic response observed on the short timescale (Figures 2 and 3).

To discern a possible involvement of dopamine on NAc cell firing on the long timescale we re-examined the activity of neurons tested under RACLO, SCH or RO across the longer time domain. NAc neurons previously tested under RACLO and classified as phasically active under the short time interval (Fig. 4; n = 9 I-type and n = 6 E-type) exhibited no change in overall firing rates across the long timescale due to repeated stimulations prior to drug delivery. Moreover, as with the short duration window, RACLO had no significant effect on overall firing rate in this population of neurons ($F_{(1, 28)} = 0.02$, $p = 0.12$). Another subset of cells previously classified as I-type (n=7) or E-type (n=5) and tested with SCH also showed no change in activity prior to drug delivery across the long timescale. However, SCH significantly increased long-term unit activity in half the population of cells (n = 6/12 neurons; $F_{(1, 10)} = 3.44$, p Q 0.05). Finally, abolition of vesicular dopamine release by RO was tested in a population of neurons (n=7) previously characterized as I-type under the short duration interval. Interestingly, these neurons displayed gradual excitations on the long-time scale that were dramatically reduced by RO, and this inhibition in firing was further decreased by a subsequent bicuculline injection ($F_{(4,20)} = 3.69$, p Q 0.05). Collectively, the effects of dopamine antagonists on overall firing rates are consistent with prior reports (Kiyatkin and Rebec, 1999) and, together with the RO data, lend support to the notion that long-term changes in cell firing may be influenced by dopamine.

Discussion

The present study is the first to measure on a subsecond time scale both phasic dopamine release and neuronal firing at a single location in the NAc of awake, unrestrained rats. To accomplish this, we adapted technology developed to examine cell firing and catecholamine release in anesthetized preparations for use in freely moving animals (Armstrong-James et al., 1980). Both with a stimulus train that rats will self administer and with one that mimics the naturally occurring bursts of dopaminergic neurons, changes in unit activity time-locked to MFB stimulation occurred simultaneously with dopamine release. The predominant time-locked response of NAc neurons was an inhibition of firing that coincided temporally with dopamine concentration changes. However, pharmacological evidence suggests that $GABA_A$ receptors rather than D1-like or D2-like receptors may be responsible for these inhibitions of NAc firing. While this study does not reveal dopamine-mediated, time-locked changes in unit activity that are associated with dopamine release, dopaminergic modulation of the long term firing rate of subsets of NAc cells on a minute-to-minute time scale was observed, and transient dopamine concentrations may be critical in this action.

Measurements of dopaminergic effects on the firing rate of NAc neurons is expected to differ from those in brain slices or isolated cells because those preparations lack neurotransmitter tone resulting from ongoing neural activity (Seamans and Yang, 2004). The overall view from those studies is that dopamine is a modulator of ongoing activity (Nicola et al., 2000). In intact, but anesthetized, animals rapid changes in unit activity have been reported following dopamine release evoked by MFB stimulation. With stimulations similar to the longest that we used, Williams and Millar found that striatal units were excited while dopamine was elevated, and the elevated firing continued for 10s of seconds after the stimulation (Williams and Millar, 1990).

With longer stimulations that evoked greater dopamine release, the excitation reverted to an inhibition. These responses were suppressed following dopamine depletion by synthesis inhibition. Excitation, mediated by D1 receptors, was also observed by Gonon within 200 ms of the stimulation in a subpopulation of striatal neurons in urethane anesthetized rats (Gonon, 1997). In contrast to these previous reports, in the present study of NAc unit activity, the predominant response was an inhibition. Indeed, a large proportion of the cells that were excited by MFB electrical stimulation met criteria for antidromic activation (Wolske et al., 1993), and were therefore not further examined.

Anesthesia is known to dramatically dampen neuronal excitability, and thus differences in unit activity in awake animals are expected. For example, larger stimulation currents are required in the anesthetized preparation to evoke similar dopamine release (Garris et al., 1997). Our results are similar to those of Kiyatkin and Rebec in awake rats who examined the effects of iontophoretic application of dopamine (Kiyatkin and Rebec, 1999). Both in the striatum and NAc, dopamine had little effect on baseline unit activity unless delivered in the presence of glutamate. Dopamine (at elevated concentrations) potentiated the action of glutamate, primarily via a pathway inhibited by a selective D1 antagonist. However, our experimental design did not directly probe glutamatergic activation or dopamine-glutamate interactions.

Because the inhibition in unit firing evoked by stimulation of the MFB was not blocked by dopamine receptor antagonists or by elimination of dopamine release following inhibition of the vesicular monoamine transporter, we sought another neurotransmitter candidate. GABAergic neurons from the VTA are known to traverse within the MFB along with ascending dopamine systems (Carr and Sesack, 2000). Thus, in addition to inhibitory circuitry provided by local circuit neurons (Smith and Bolam, 1990), NAc neurons are also subject to extrinsic GABA

inputs from other brain regions. A similar GABAergic inhibition was found during electrical stimulation of the VTA while monitoring unit activity in the prefrontal cortex (Pirot et al., 1992). Surprisingly, the duration of bicuculline-sensitive inhibition of unit activity in the NAc matches the time course of the dopamine concentration changes. This may arise because the extracellular lifetime of GABA before uptake via the GABA co-transporter (rGAT1) is similar to that of dopamine. rGAT1, like the dopamine transporter (DAT), has a turnover rate approximately three orders of magnitude slower than the decay rate of synaptic events (Mager et al., 1996).

The long term changes in unit activity following evoked dopamine release or its inhibition are consistent with changes in activity mediated by G-protein coupled receptors. Unlike classical neurotransmitters whose receptors directly control ion channels, dopamine receptors are G-coupled proteins that operate through second messengers. The receptor responses can have a slow onset and a prolonged effect. For example, D-2 mediated autoinhibition of pulsatile dopamine release requires 700 ms for full expression and exhibits a duration of more than 10 s (Kennedy et al., 1992; Phillips et al., 2002). Similarly, D1 receptor-mediated inactivation of sodium currents is slow both in onset (Yan et al., 1999) and time to reach a steady-state (~ 15 min, Hayashida and Ishida, 2004). The time scale of these changes in unit activity is similar to those recently observed in the prefrontal cortex following VTA stimulation (Lavin et al, 2005).

As these results show, the use of a single electrode to monitor both a neurotransmitter and single-unit activity provides unique information about neurotransmission on a subsecond timescale. The measured events are both obtained from a volume of tissue that has dimensions of a few micrometers. Dopamine, an extrasynaptic messenger, diffuses from its release site to target receptors (or the electrode) but the DAT constrains its spread to the local area (Cragg and Rice,

2004). The local concentration that is experienced by extrasynaptic receptors is measured directly. Similarly, the unit responses are restricted to less than 40 Km of the cell body because of the limited spread of the electric field (Williams and Millar, 1990). Despite the constrained sampling area, in this study the timelocked changes in unit activity did not appear to be dopamine mediated. Because dopamine is a neuromodulator of the effects of other neurotransmitters that are directly responsible for the control of unit activity during normal behavior, noncontingent stimulation of dopamine neurons may simply not engage the remainder of the circuitry that is normally involved in neuronal encoding, for example during goal-directed behavior.

In contrast to stimulation experiments, naturally occurring dopamine transients occur as part of a complex set of accompanying neuronal changes. For example, when triggered by the inputs to the brain reward circuitry, rapid dopaminergic cell firing occurs (Montague et al., 1996; Schultz et al., 1997; Berridge and Robinson, 1998; McClure et al., 2003), and, as we have shown, such behaviors are accompanied by dopamine transients in the NAc (Robinson et al., 2002; Phillips et al., 2003; Stuber et al., 2005). Although their role is still unknown, we suggest three possibilities. As seen with the long term changes seen in this work, dopamine transients could simply contribute to steady-state dopamine levels that modulate ongoing activity mediated by other neurotransmitters. A second possibility is that dopamine transients modulate rapid changes in unit activity only when the other circuit components are engaged. Indeed, fast intracellular responses mediated by dopamine in the PFC or NAc may not directly increase cell firing but may make neurons more susceptible to afferent activation (Goto and O'Donnell, 2001; Beckstead et al., 2004). The third possibility rests on the fact that transient bursts are the simplest way to achieve high extracellular dopamine concentrations. This would allow activation of a

subpopulation of low affinity receptors that are normally unaffected by tonic dopamine levels.

Future experiments with controlled behaviors will investigate these possibilities.

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Figure Legends

Figure 1. Combined electrochemistry and electrophysiology in the nucleus accumbens. (A) Dopamine release in the NAc evoked by electrical stimulation of the MFB delivered at the time indicated by solid bar. The color representation shows all the voltammetric data and the oxidation currents associated with dopamine (green). A dopamine-specific cyclic voltammogram obtained at the peak of the electrically-evoked release is shown to the right (vertical dashed line). The trace above the color plot is the current at the potential where dopamine is oxidized, corrected for ionic changes, shows the rapid nature of dopamine release and uptake. (B) Representative perievent raster of accumbal unit activity and associated histogram centered on the electrical stimulation (biphasic pulses, 0.4 s duration, 60 Hz, ± 125 KA; green dashed line at time 0). Each tick on the raster plot represents an extracellular recorded action potential (average waveform and calibration shown to the right). For this particular location, the firing rate of the neuron was inhibited when electrically-evoked dopamine release was detected. The overlaid red trace displays the evoked dopamine signal averaged from the 10 trials shown. Bin width: 200 ms for both measurements.

Figure 2. Classification of accumbal firing patterns obtained simultaneously with noncontingently evoked release of dopamine. Composite perievent histograms show three different neuronal populations constructed ± 15s around medial forebrain bundle electrical stimulations (24 pulses, 60Hz, 125 KA; green dashed line at time 0), which consisted of summing data from each individual neuron over individual trials. The overlaid trace (red) shows the time course of average extracellular dopamine concentration changes measured at the same loci where neurons were recorded following the stimulation (U = unaffected; I = inhibited; I/E =

inhibited/excited). Note that the stimulation-induced inhibition closely matched the release of dopamine and the compound nature of the response of I/E-type neurons. Bin width: 200 ms for both measurements.

Figure 3. Accumbens firing patterns and dopamine outflow obtained following a dopamine neuron burst-like stimulation. (A) The overlaid trace (red) shows the time course of average extracellular dopamine concentration changes measured at the same loci where neurons (U = unaffected; I. = inhibited; E = excited) were recorded following a dopaminergic neuron, burst-like MFB stimulation as shown by the composite perievent histograms (6 pulses, 30 Hz, 125 KA; green dashed line at time 0). This stimulation yields two categories, I-type and E-type cells, whose time-locked changes closely match the duration of the evoked dopamine signal. No I/E-type cells are observed following application of this more physiologically relevant stimulation protocol. (A_1). A majority of E-type cells (67%) were antidromically driven as evidenced by the invariable latency between each pulse in the train (*) and the directly activated action potential (•). (B) The color plot depicts a non-stimulated DA transient recorded at the time indicated by the asterisk and the transient evoked by a VTA dopamine neuron burst-like stimulation (6 pulses, 30 Hz; black bar). The trace above the color plot is the current at the potential where dopamine is oxidized, corrected for ionic changes. The amplitude of the non-stimulated transient is similar to that of the electrically evoked change. (B $_1$) The correlation coefficient (r2 = 0.75) for the comparison of the two overlaid voltammograms (stimulated shown in black, non-stimulated shown in red) indicates that the current detected at the first vertical dashed line (asterisk) is due to dopamine oxidation.

Figure 4. Lack of effect of blockade of dopamine receptors on inhibitory and excitatory responses time-locked to dopamine neuron burst-like stimulation. (A) The time-locked inhibition of firing in I-type neurons is not different between pre- (top) and post-drug (bottom) following blockade of D1 receptors by SCH23390 (SCH, 40 Kg kg^{-1}; left column). Blocking D1 receptors does not modify the amplitude of electrically evoked release (red trace) following the stimulation (green dashed line at time 0) obtained at the locations where this population of I-type
neurons was obtained. A similar lack of effect on patterned firing is observed following application raclopride (Raclo, 80 Kg kg^{-1}), a D2 receptor antagonist. However, Raclo dramatically increases electrically evoked release. (B) No differences were observed either on the excitatory profile of E-type cells or on the amount of dopamine release after SCH treatment (left column). While the excitation was unchanged by Raclo treatment, dopamine release was again increased (right column). Bin width: 200 ms for both measurements, all drugs were given intravenously.

Figure 5. Inhibitory responses are independent of vesicular dopamine release but modulated by $GABA_A$ receptors. The top composite perievent histogram shows that medial forebrain bundle stimulation (green dashed line) releases dopamine (red line) and decreases accumbal firing time-locked to the train (n = 7). Blockade of action potential-driven vesicular dopamine release with RO4-1284 (1 mg kg^{-1}, RO) abolishes detectable release and decreases background firing rates, but does not eliminate the inhibition of firing caused by the stimulation
(middle). When GABA receptors are blocked by bicuculline (BIC; 2 mg kg^{-1}) in the presence of RO, a time-locked excitation consistent with synaptic activation is uncovered, while background

firing is further decreased and dopamine release remains undetectable (bottom). Bin width: 200
ms, all drugs were delivered intravenously.

Figure 6. Electrical stimulation modifies accumbal firing rate on prolonged time scales.

Integrated firing rate histogram of the activity of 3 different neurons in the NAc (waveforms at

right) obtained from representative animals receiving noncontingent electrical stimulations of the
medial forebrain bundle (24 pulses, 60 Hz, 125 KA) that consistently released dopamine over

entire sessions of approximately 15 min. Vertical ticks on top indicate times of stimulation

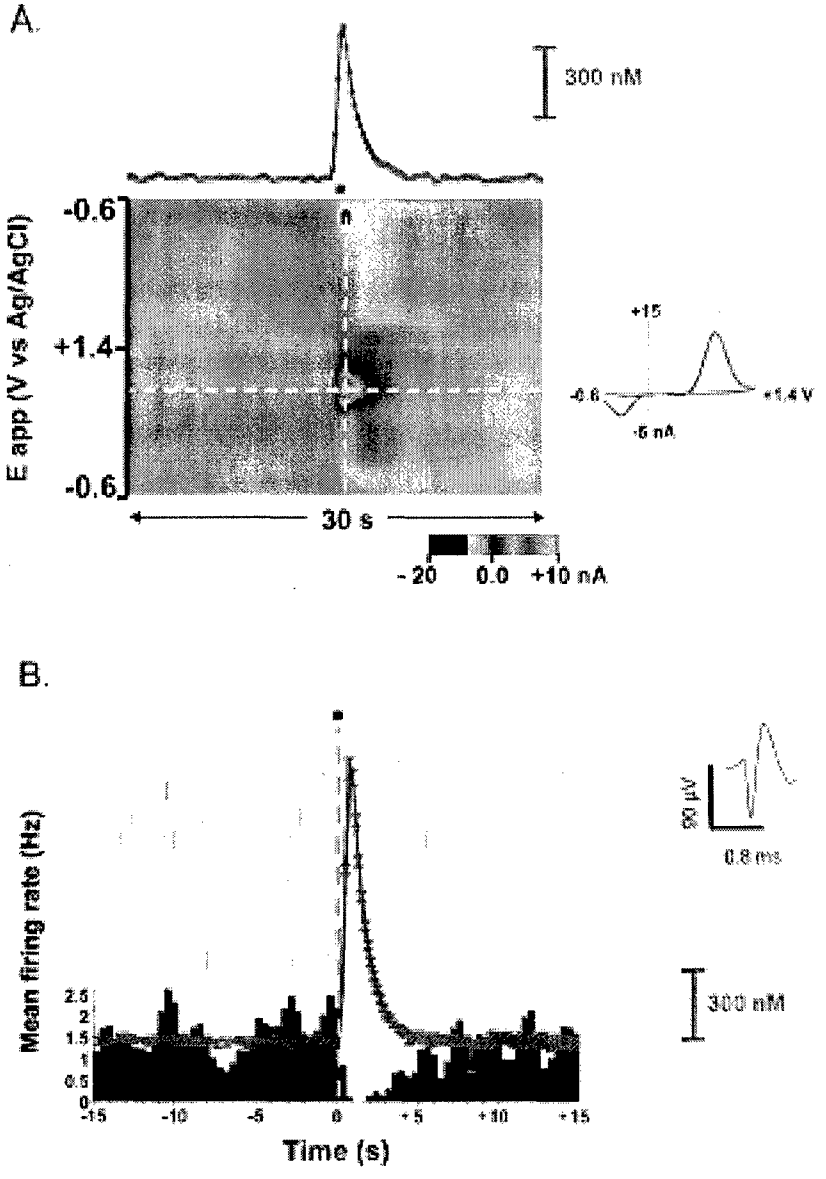
delivery. Neurons were either unaffected (top stripchart) or progressively excited (middle

stripchart) or inhibited (bottom stripchart) by repeated stimulations. Bin width: 10
s

Table 1. Changes in NAc neuron activity following MFB electrical stimulation

Stimulation	Neuron population mean frequency (Hz)			
	U-type	I-type	E-type	I/E-type*
6p 30 Hz	n = 26	n = 40	n = 14	n = 0
Baseline	1.24 ± 0.4	1.18 ± 0.4	2.25 ± 0.7	-
Response	1.28 ± 0.3	0.41 ± 0.1	3.41 ± 0.2	-
Recovery	1.36 ± 0.4	0.67 ± 0.2	2.47 ± 0.4	-
24p 60 Hz	n = 24	n = 46	n = 0	n = 12
Baseline	2.02 ± 0.9	0.83 ± 0.1	-	0.54 ± 0.2
Response	1.89 ± 0.6	0.36 ± 0.1	-	0.46 ± 0.1 1.21 ± 0.3
Recovery	1.97 ± 0.6	0.44 ± 0.2	-	0.77 ± 0.3

*Response components are separated into the short inhibition (top) and the delayed excitation (bottom). Data are means ± SEM.



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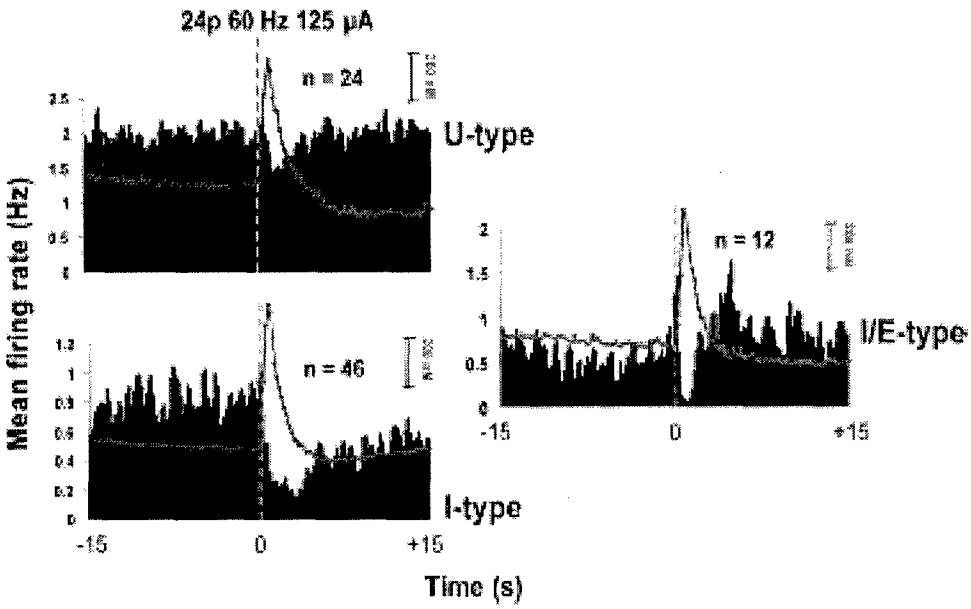

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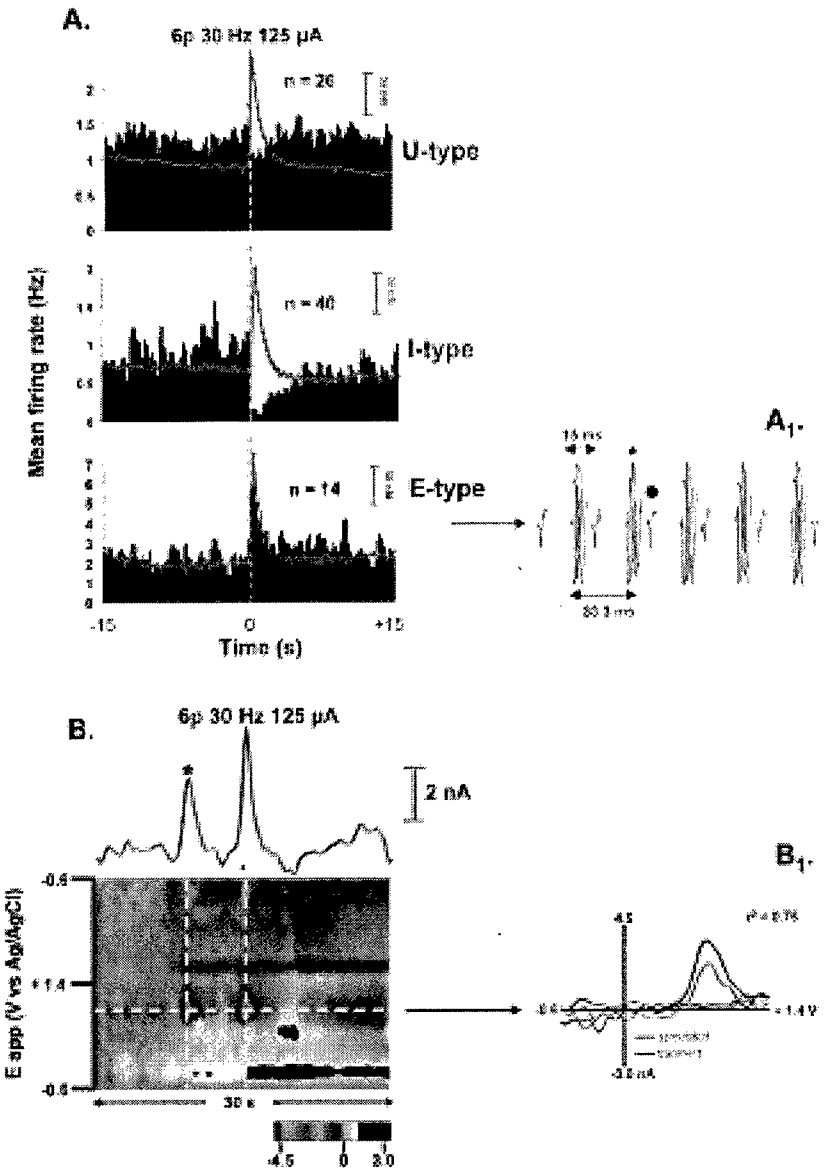

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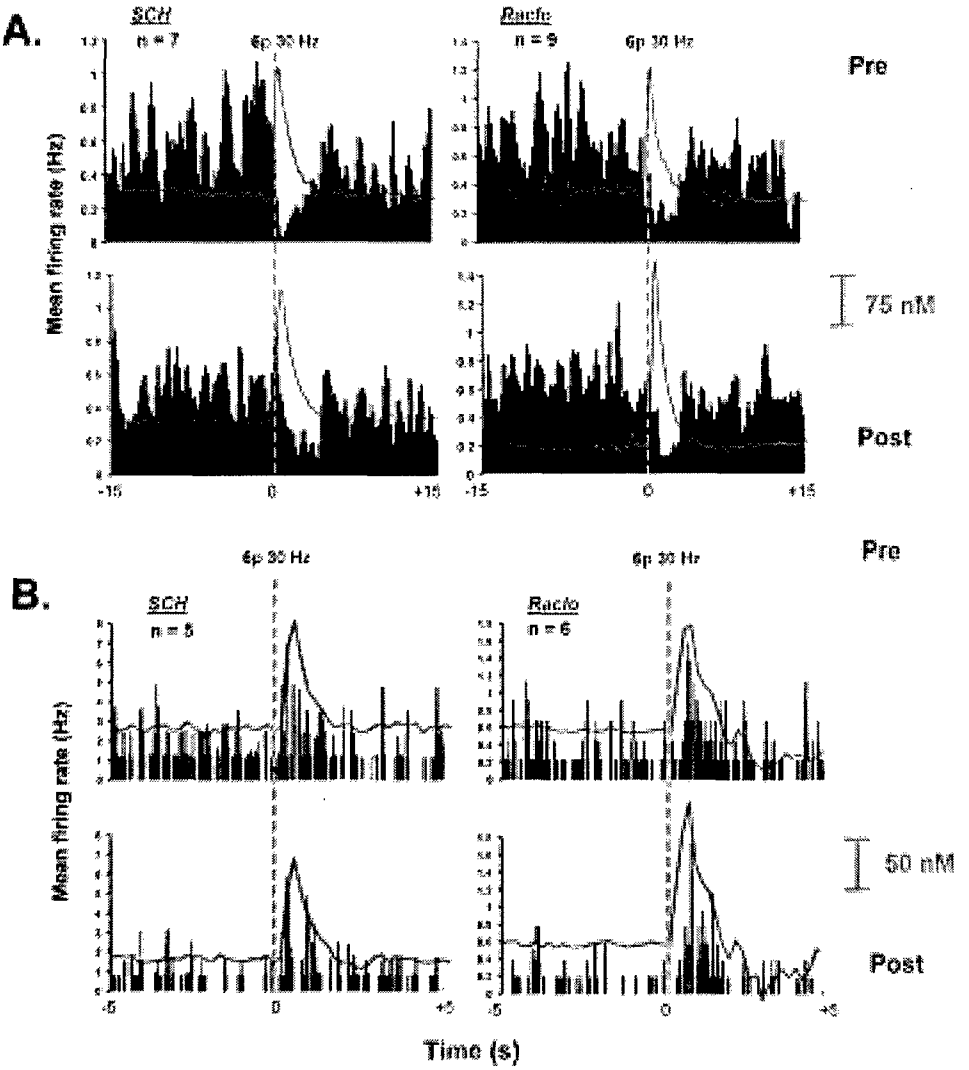

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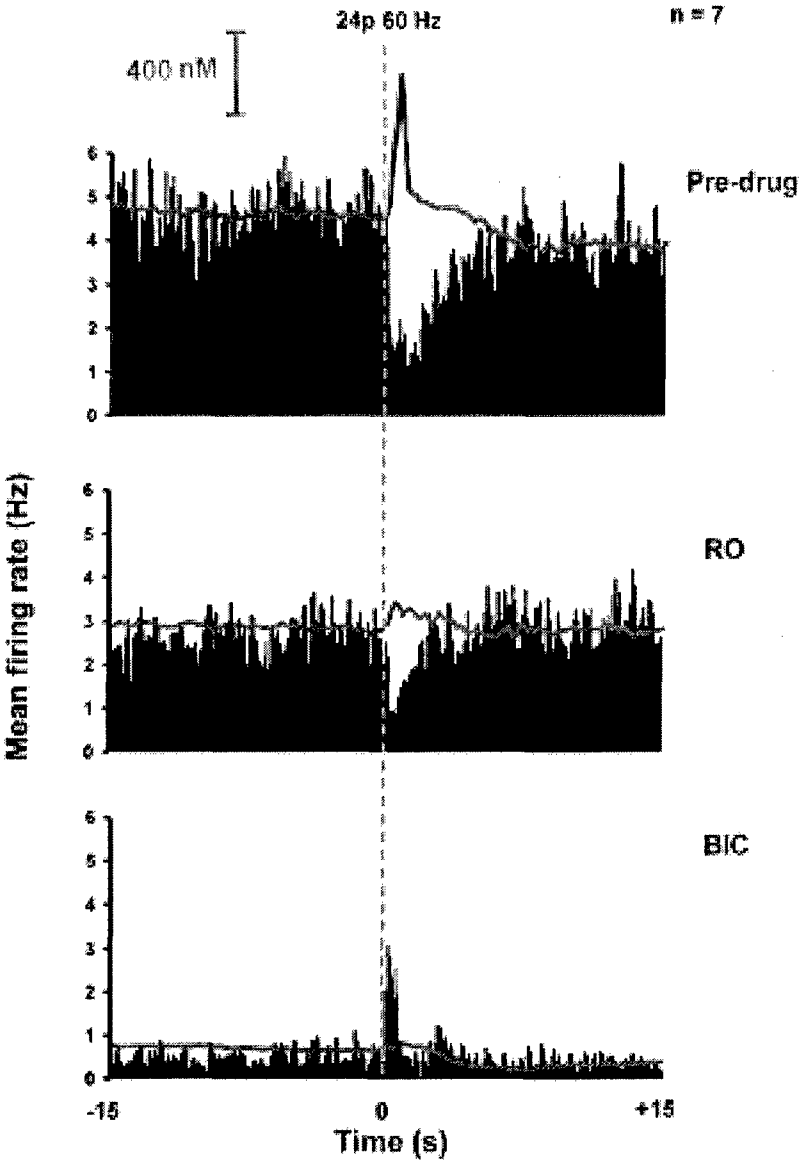

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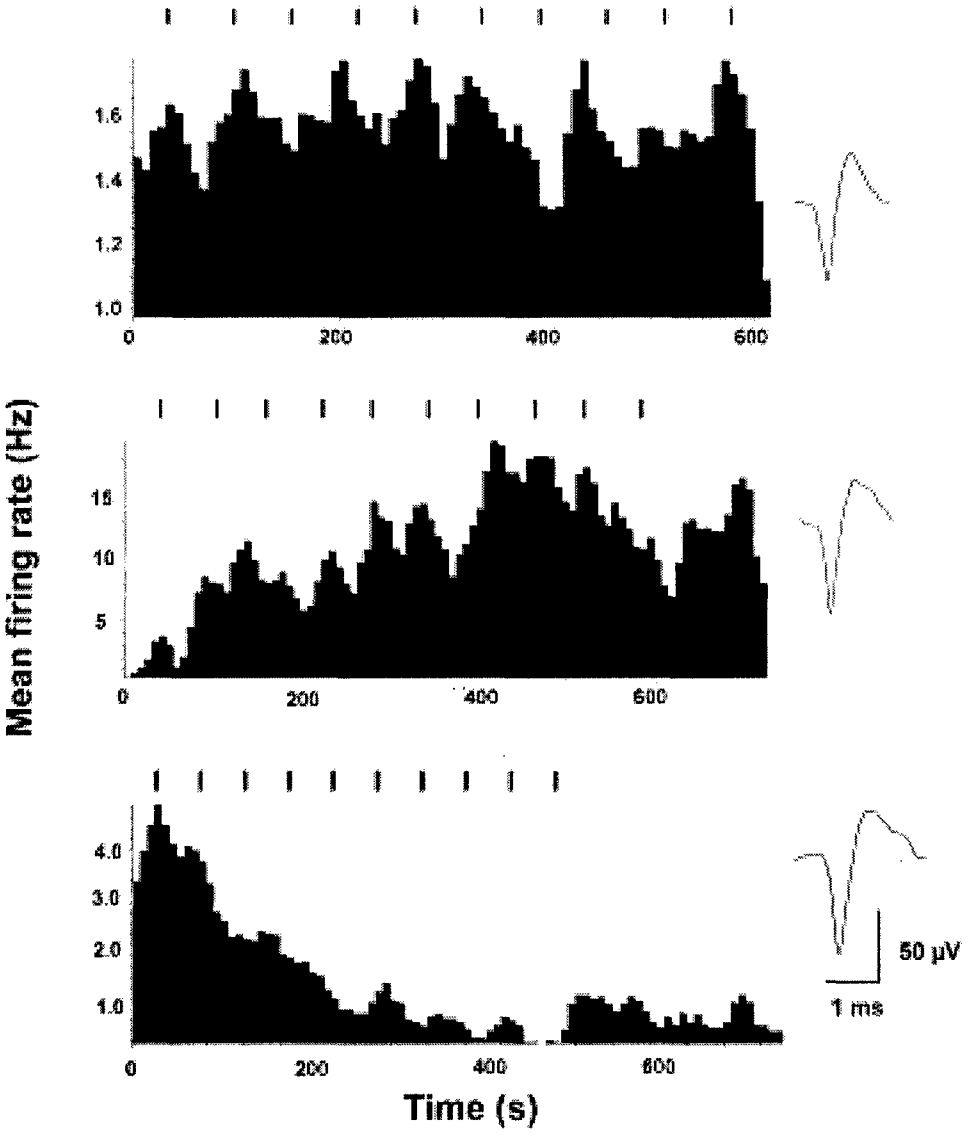

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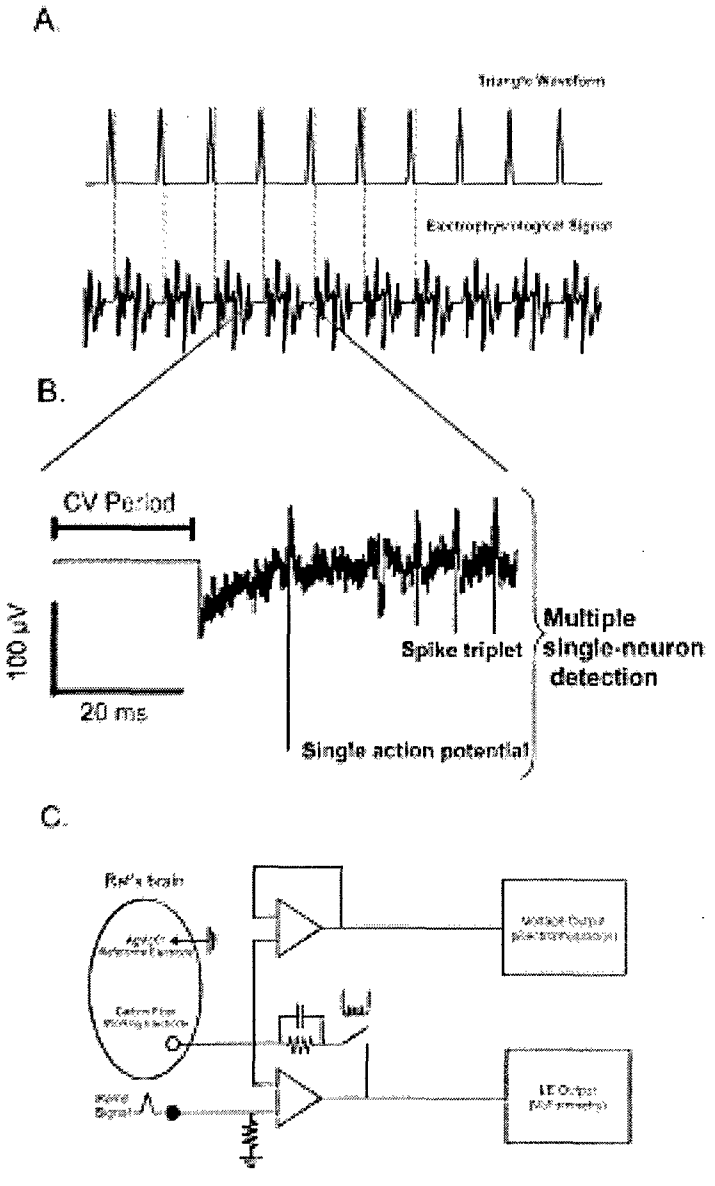

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"PASSIVE STABILIZATION" OF STRIATAL DOPAMINERGIC TONE ACROSS THE PRECLINICAL DENERVATION RANGE OF PARKINSON'S DISEASE: A THEORETICAL STUDY

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ABSTRACT

In Parkinson's disease, compensatory normalization of striatal dopaminergic tone is thought to prevent the emergence of cardinal symptoms until the loss of nigrostriatal dopaminergic neurons is extensive. However, the central adaptation postulated to maintain tone during the preclinical phase, up-regulated dopamine release by surviving neurons, has been difficult to demonstrate unequivocally. Here we use a finite difference model of brain extracellular dopamine to examine theoretical relationships between dopaminergic tone and denervation. The model incorporates release, uptake and diffusion, the primary determinants of extracellular dopamine levels. Unexpectedly, 0 to 99% denervation did not change dopaminergic tone after simulations stabilized. Inspection of the spatial profile of dopaminergic tone revealed that, shortly after release, dopamine pools in non-innervated elements, safe from clearance by the dopamine transporter. A second dopamine clearance term, described by a first-order rate constant (k) and mimicking non-dopamine transporters, was then incorporated into the model. Values of k between 0.05 and 0.1 s^{-1}, similar to that documented in dopamine transporter knock-out mice, generated the lesion profile of dopaminergic tone established by microdialysis in 6-hydroxydopamine-lesioned rats. These simulations form the theoretical framework for a new hypothesis of compensatory adaptation during the preclinical phase of Parkinson's disease called "Passive Stabilization", whereby dopaminergic tone is maintained without active changes in release or uptake. Results also support non-dopamine transporters as a drug target for treatment of Parkinson's disease.

INTRODUCTION

Parkinson's disease is a debilitating neurodegenerative disorder associated with nigrostriatal dopaminergic denervation (Lang and Lozano, 1998;Olanow and Tatton, 1999). Observation that cardinal symptoms of resting tremor, rigidity and akinesia do not emerge until striatal dopamine content loss exceeds ~80% led to the postulate that an adaptive capacity of the nigrostriatal dopaminergic system maintains motor function during the preclinical phase (Hornykiewicz, 1966;Hornykiewicz and Kish, 1987). However, the precise nature of this compensation has remained an enigma for almost forty years. Microdialysis in the 6-hydroxydopamine-lesioned rat indicate that normalization of dopaminergic tone, the ambient concentration of brain extracellular dopamine, is the primary adaptation (Robinson and Whishaw, 1988;Zhang et al., 1988;Abercrombie et al., 1990;Parsons et al., 1991;Robinson et al., 1994). The prevailing view is that increased dopamine release by surviving neurons drives dopamine diffusion from innervated to denervation regions, thus supplying dopaminergic tone throughout the striatum (Zigmond et al., 1990;Zoli et al., 1998).

Despite the fact that up-regulated dopamine release is proposed as the central compensatory mechanism maintaining striatal dopaminergic tone, evidence is equivocal. Indirect indices of release, e.g., dopamine metabolites, dialysate dopamine and evoked dopamine overflow, when normalized to tissue dopamine content, increase only after dopamine depletion exceeds ~50% (Melamed et al., 1980;Zigmond et al., 1984;Zhang et al., 1988;Snyder et al., 1990). More direct methods, e.g., combining voltammetry with kinetic analysis (Garris et al., 1997;Bergstrom and Garris, 2003) or

"pseudo-one pulse" stimulation (Bezard et al., 2000), fail to demonstrate any adaptation altogether. These observations raise the intriguing question: if dopamine release is not up-regulated, then how is dopaminergic tone normalized? One possibility is down-regulation of dopamine uptake, achieving the same end as increased release. Although dopamine uptake may down-regulate following severe lesions (Uhl et al., 1994;Joyce et al., 1997;Rothblat and Schneider, 1999), there is no evidence for this adaptation following partial lesions (0-~80%)(Zigmond et al., 1984;Altar et al., 1987;Dentresangle et al., 2001). Alternatively, firing rates for surviving nigrostriatal dopaminergic neurons could increase or change pattern. However, electrophysiological studies document only one adaptation, increased burst firing only at dopamine lesions greater than 95% (Hollerman and Grace, 1990). Thus, the basis for normalized dopaminergic tone is not established presently.

Here we investigate theoretical relationships between dopaminergic tone and denervation using a one dimensional, finite difference model incorporating dopamine release, uptake and diffusion (Schmitz et al., 2001;Venton et al., 2003). One portion of the striatal dopaminergic field was reconstructed by converting a confocal image of tissue stained for the dopamine transporter into innervated and non-innervated volume elements. Only innervated elements release and uptake dopamine; diffusion occurs throughout. Denervation is simulated by removing innervated elements without altering properties of release and uptake in remaining elements. Addition of a second extracellular clearance mechanism, mimicking non-dopamine transporters in the striatum, generated a lesion profile for dopaminergic tone consistent with previous microdialysis experiments. Therefore, these calculations support the hypothesis that dopaminergic tone is passively maintained across the preclinical lesion range of Parkinson's disease

without compensatory adaptation of release or uptake, primary determinants of extracellular dopamine levels in brain.

METHODS

One release event is modeled by a step increase in concentration; uptake is modeled by Michaelis-Menton kinetics. used for simulating striatal dopaminergic tone following dopaminergic terminal denervation. Extracellular space is divided into volume elements. Dopamine is free to diffuse between elements and is added by release or removed by uptake. The same equations used to curve fit voltammetric signals in lesion studies (Wu et al., 2001;Bergstrom and Garris, 2003) are employed. Parameters for DA release and uptake were taken from voltammetric studies in intact animals. Tonic dopaminergic signaling was modeled by random DA release events occurring at an overall rate of 5 Hz. Release in each volume element was also independent of all others. This scheme mimics dopaminergic neuron electrophysiology during tonic firing (Grace, 2000). Release events are identified in the left graph by ticks under the x-axis.

Statistical Analysis

Diffusion between volume elements is governed by equation 1:

$$\Delta DA_{diffusion} = \frac{1}{2} DA_{j-1} + \frac{1}{2} DA_{j+1} - DA_j . \qquad (1)$$

where DA_{j+1} and DA_{j-1} are the DA concentrations in the neighboring elements on the previous time step. Dopamine uptake will be calculated by Michaelis-Menton kinetics shown in equation 2:

$$\Delta DA_{uptake} = \frac{V_{max}[DA]}{K_m + [DA]} \Delta t \qquad (2)$$

where V_{max} is the maximal velocity of uptake and related to the total number of DA transporters, and K_m is inversely related to affinity of the transporter for DA. Dopamine release is modeled by a step increase in volume element concentration arising from each stimulus pulse. This release term is called the concentration of DA elicited per stimulus pulse ($[DA]_p$).

Time average, spatially averaged, repetitions, mean±SEM where n is the number of repetitions.

RESULTS

Finite difference model of extracellular dopamine regulation

Figure 1 describes the conceptual basis for the finite difference model used in the present study (Venton et al., 2003). The micrograph displayed in the middle is a confocal image of a strip of striatal tissue stained fluorescent green for the dopamine transporter, a marker for dopaminergic neurons. Positively stained striatum is assigned an "innervated" volume element exhibiting properties for dopamine release and uptake (hatched squares below micrograph). One dopamine release event is modeled by the step increase in dopamine concentration generated by one action potential during simulated basal firing of dopaminergic neurons. Dopamine uptake is modeled by the dopamine transporter using Michaelis-Menton kinetics. Parameters for release and uptake were identical for all innervated volume elements and taken from voltammetric measurements of electrically evoked dopamine levels in the intact, non-lesioned rat striatum (Venton et al., 2003). "Non-innervated" elements assigned to unstained tissue (open squares) do not exhibit dopamine release and uptake properties. Diffusion operates in all elements.

On top of the confocal image in Figure 1 are two graphs showing the temporal profile of dopamine concentration for individual innervated (left) and non-innervated (right) volume elements. Release events are identified in the left graph by tick marks under the x-axis. The overall rate for the random release events is 5 Hz. Release events are independent in all innervated elements. These characteristics are consistent

with dopaminergic neuron electrophysiology during basal firing (Grace, 2000). Concentration changes in the innervated element are ragged due to release events and dopamine diffusing in from adjacent elements. In contrast, dopamine concentration changes are smoother in the non-innervated element over the same time period (right graph). Although sometimes higher due to release or lower due to uptake, the spatially averaged concentration in the innervated volume element is similar to that in the non-innervated element. To simulate nigrostriatal dopaminergic lesions, innervated elements were randomly converted into non-innervated elements until the desired denervation level was reached (see strips of volume elements under the confocal image). For each set of simulations, the preceding, less denervated pattern was used to create the subsequent, more denervated pattern. Thus, a volume element denervated at 80% innervation, for example, would remain denervated for the remaining lesions. Dopamine release and uptake properties for the surviving innervated elements were not altered.

Simulated relationships between dopaminergic tone and denervation

Figure 2A shows the temporal profile for dopamine concentration at innervation levels of 100% (intact), 80, 60, 40, 20, 10, 5 and 1% beginning at simulation time zero. All simulations described in the present study begin at a dopamine concentration of zero. Each concentration point is spatially averaged across the middle 100 μm of the striatal tissue strip. The middle portion was selected to avoid edge effects in the simulation. The top curve, fastest to rise, is 100% innervation, and the bottom curve,

slowest to rise, is 1% innervation. With the exception of 1% innervation, all lesions rapidly reached a same steady-state concentration of approximately 30 nM and remained at this level for the duration of the simulation. Even 1% innervation reached this concentration eventually between 200 and 300 s. An expanded view of the temporal profiles is shown in Figure 2B. Steady-state dopamine levels for all lesions are shown in Figure 3B. To avoid artifacts from random release events and lesion pattern, concentrations were averaged across 1 s and across 10 independent simulations. Consistent with temporal profiles, steady-state dopamine concentration is similar across the innervation range between 1 and 100%. This result provides theoretical evidence for the postulate that dopaminergic tone is maintained across the preclinical denervateion range in Parkinson's disease without compensatory adaptation of dopamine release and uptake. Rrather surprisingly, dopaminergic tone did not drop at severe lesions (>~80% innervation) as expected based on microdialysis measurements in 6-hydroxydopamine lesioned rats (Robinson and Whishaw, 1988;Abercrombie et al., 1990) .

Inspection of the spatial profile of extracellular dopamine provides one possible explanation for the failure of dopaminergic tone to drop at severe lesions. In the simulations shown in Figure 3, two innervated elements were fixed 100 μm apart *a priori*. Assuming a uniform distribution of innervated volume elements, this inter-element distance corresponds to 1% innervation. This separation was also selected to emphasize the importance of non-innervated volume elements. The different curves were collected at various times to canvass the rise and plateau portions of the temporal profile shown in Figure 2. Both innervated elements recently released dopamine at 1.2

s. At this time, little dopamine accumulates in intervening non-innervated elements. Although no release events occurred recently for curves at 6.9, 13.2 and 22.1 s, a gradual concentration increase is apparent in the non-innervated elements. The steady-state level is achieved by 72.5 s. Pooling occurs in non-innervated elements because there is no clearance mechanism. For removal from extracellular space, dopamine must diffuse back into an innervated element, which represents a dopamine sink in addition to a dopamine source. In fact, due to uptake, curves at 6.9, 13.2 and 22.1 s show lower dopamine levels in and around the innervated elements at either end compared to intervening non-innervated elements. The curve at 72.5 s shows the two innervated elements acting either a dopamine source (left) or sink (right). Thus, dopaminergic tone is normalized even after severe denervation, because dopamine pools in the predominant non-innervated elements where it is safe from dopamine uptake.

Addition of second dopamine clearance mechanism

Simulations calculated from the original finite difference model clearly demonstrate how readily dopaminergic tone is maintained without compensatory changes in dopamine release and uptake. To be sure, passive normalization appears quite potent. Unfortunately, simulations failed to account for the expected drop in extracellular dopamine at severe lesions. Efforts were next directed at finding conditions that would capture the experimentally determined lesion profile of dopaminergic tone: normal until ~80% denervation following by a decrease thereafter.

Non-dopamine transporter clearance of extracellular dopamine in the striatum may provide one potential solution. Perhaps minimally effective in intact tissue due to the high affinity and abundant dopamine transporter, these other clearance mechanisms might become efficient collectively under conditions of severe dopamine denervation. Therefore, an additional extracellular clearance mechanism, described by first-order kinetics and operating in all volume elements, was incorporated into the finite difference model. As shown in Figure 4, addition of the second dopamine clearance mechanism resulted in the expected drop in dopaminergic tone at the most severe lesions. Similar to Figure 2C, these values were temporally averaged across 10 independent simulations. A k between 0.05 and 0.25 s^{-1} gave the most reasonable lesion profiles. At these values, dopaminergic tone was flat until less than ~20% innervation and then precipitously fell at lower innervations. Moreover, at innervations greater than ~20%, extracellular dopamine levels were similar to that without the additional clearance mechanism.

Because a k of 0.1 s^{-1} generated the expected lesion profile, this value was used for subsequent simulations. Figure 5 shows temporal profiles of dopaminergic tone with respect to lesion degree. Similar to Figure 2, results were spatially averaged. In sharp contrast to simulations without the second clearance mechanism, all curves reached steady state quickly, within approximately 20 s. Steady-state levels did not change for the duration of the simulation (300 s) for any innervation. The lesion profile, evident in the averaged results shown in Figure 4, is also apparent in the temporal profiles. Figure 6 shows spatial profiles collected at different times for 1% denervation with two innervated volume elements fixed 100 μm apart. In general, spatial profiles are similar to those without the second clearance mechanism (Fig. 3). Although approaching a lower

averaged concentration, dopamine similarly pools in non-innervated volume elements. The one notable exception is that dopamine concentration in the middle of the non-innervated elements does not exceed that in or adjacent to innervated elements. This observation demonstrates the effects of the second clearance mechanism and directly results in lowered dopaminergic tone at severe denervation.

Figure 7A compares spatial profiles for different innervation levels. To reduce clutter, only three innervations, 1, 10 and 100%, are displayed. Denervation was random, and, after dopamine concentration had stabilized, curves were temporally averaged over 1 s to avoid random release artifacts. Dopamine concentrations for the three innervation levels are, in general, consistent with lesion profiles shown in Figure 4. The absolute concentration range across the 100 μm tissue strip, defined as the difference between highest and lowest dopamine concentration, is typically greatest at 100% and lowest at 1% innervations, with 10% innervation in between but closer to that for 100% innervation. More insight in gleaned by examining normalized concentration range plotted for each time point over a 6 s bin as shown in Figure 7B. Normalized range is defined as absolute range divided by the spatially averaged concentration. This calculation compares concentration changes relative to baseline concentration. When normalized, range is typically greatest for 100% innervation but similar overall for 1 and 10% innervation. Moreover, normalized range changes markedly over time dependent upon innervation. Instantaneous range changes for 100% innervation deviate the greatest, characterized by the most spikes and the greatest amplitude spikes. Spikes are fewer and smaller for 10% innervation, and almost non-existent, but clearly of low amplitude and broader, for 1% innervation. These results suggest that

instantaneous dopamine concentration changes, while markedly fluctuating in intact tissue, passivate with increasing denervation.

Simulated dopaminergic tone in the lesioned rat and monkey

DISCUSSION

The present study used a theoretical approach to address the enigma of how striatal dopaminergic tone normalizes across the preclinical denervation range of Parkinson's disease. The critical issue is if dopamine release is not up-regulated, then another compensatory mechanism must be invoked. A finite difference model, incorporating dopamine release, uptake and diffusion and extracellular clearance by non-dopamine transporters, generated the lesion profile for dopaminergic tone experimentally established by microdialysis. These results suggest that dopaminergic tone can be normalized passively without compensatory changes in dopamine release or uptake.

Up-regulated dopamine release

Profound changes in dopaminergic neurotransmission emerge at ~50% denervation and progress with greater lesion degree. These include, when normalized to tissue dopamine content, increases in dialysate dopamine (Zhang et al., 1988;Abercrombie et al., 1990), electrically evoked dopamine overflow (Stachowiak et al., 1987;Snyder et al., 1990), dopamine synthesis (Zigmond et al., 1984;Altar et al., 1987;Wolf et al., 1989) and dopamine metabolites (Melamed et al., 1980;Altar et al., 1987). The prevailing view is that increased dopamine synthesis sustains while increased dopamine metabolites reflect up-regulated dopamine release (Zigmond et al., 1990). Dopamine transporter loss on lesioned dopaminergic neurons passively enhances dopamine diffusion from innervated to denervated regions and driven by up-regulated

release, because high-affinity uptake normally restricts the distance dopamine diffuses. Changes in dopaminergic neuron firing rate and postsynaptic dopamine receptors are not indicated following partial lesions, but dopamine receptor sensitization and other, non-dopamine compensation may begin to emerge during the progression towards severe denervation (Bezard et al., 2003).

In addition to what mechanism normalizes dopaminergic tone prior to ~50% denervation, another question regarding the prevailing view is how reliably do dialysate dopamine and evoked dopamine overflow evaluate release. These indices, essentially a measurement of extracellular dopamine, are generally considered indirect, because extracellular dopamine is regulated by the combination of release, uptake and diffusion (Nicholson, 1995). More direct assessment of release is provided by voltammetry combined with kinetic analysis (Wightman et al., 1988) or "pseudo-one pulse" stimulation (Garris et al., 1994). Kinetic analysis resolves voltammetric recordings of electrically evoked dopamine levels into individual components of release, uptake and temporal distortion due to brain diffusion and microelectrode response time. "Pseudo-one pulse" stimulation is less direct, but useful nonetheless. Because uptake and diffusion have little time to act during the short, high-frequency stimulus train, signal amplitude approximates release. More direct indices have failed to detect up-regulated dopamine release (Garris et al., 1997;Bezard et al., 2000;Bergstrom and Garris, 2003), thus challenging this proposed adaptation.

"Passive Stabilization" of dopaminergic tone

Simulations described in the present study form the theoretical framework for the "Passive Stabilization" hypothesis, which postulates that dopaminergic tone is normalized without compensatory adaptation of either dopamine release or uptake (Bezard et al., 2003;Bergstrom and Garris, 2003). This hypothesis was based on voltammetric measurements of electrically evoked dopamine levels in the dopamine-depleted rodent striatum. The physical principles of steady state and diffusion provide the conceptual basis for "Passive Stabilization". Dopaminergic tone is assumed to be a steady-state concentration determined by the balance between the input flux of dopamine release and the output flux of dopamine uptake. One consequence of steady state is that concentration does not vary if input and output fluxes change similarly. Therefore, because release and uptake decrease proportionally owing to co-localization on dopaminergic neurons, dopaminergic tone is passively maintained following denervation. In addition to providing a dopamine source for denervated striatal tissue (Zigmond et al., 1990), diffusion also plays the critical role by rapidly mixing dopamine released into extracellular space (Garris et al., 1994;Cragg et al., 2001).

Simulations support the validity of the essential assumption underlying "Passive Stabilization", a steady-state level of dopaminergic tone. Random dopamine release and heterogeneously innervated volume elements generate temporally averaged dopamine concentrations which are relatively constant across the entire striatal tissue strip (Venton et al., 2003). Spatially averaged concentrations are similarly consistent across the strip once dopamine levels stabilized (Figs. 2 and 5). Because diffusion is fast compared to the slow release rate of 5 Hz, sufficient mixing of extracellular dopamine occurs in between random release events. Although lesions simulations and

voltammetry have not been directly compared, the initial finite difference model well describes electrically evoked dopamine levels voltammetrically monitored in the intact rat striatum under drug-free conditions and after administration of dopamine uptake inhibitors (Venton et al., 2003), supporting the veracity of the model.

One additional clearance mechanism was incorporated to account for the drop in dopaminergic tone at severe denervation. In this new scheme, the striatum potentially contains three additional dopamine sinks, uptake by noradrenergic and serotonergic transporters (Moore and Card, 1984;Amara and Kuhar, 1993)(serotonin in chemical handbook) and glial catecholamine transporters (Russ et al., 1996;Schomig et al., 1998). Because noradrenergic and serotonergic innervations of the striatum are sparse and the glia transporter is low affinity, non-dopamine transporters have negligibly effects in intact and moderately denervated tissue. Only at more severe denervation does the second clearance mechanism compete with the high affinity and normally abundant dopamine transporter. Suitable rate constants for the second clearance mechanism were similar but slightly higher than 0.015 s^{-1}, the value determined in the striatum of dopamine transporter knockout mice (Jones et al., 1998). No data are available for other preparations. It is interesting to speculate that dopamine release decompensates at severe lesions, bringing k in line with that in transgenic mice. Although indirect indices suggest an up-regulation of dopamine release at severe denervation (Stachowiak et al., 1987;Zhang et al., 1988;Abercrombie et al., 1990;Snyder et al., 1990), no direct measurements are available. Increased dopamine release may be unlikely, particularly of the magnitude indicated by indirect indices, because very high rate constants would be required to generate the expected lesion profile of dopaminergic tone.

Simulations clearly demonstrate that released dopamine accumulates in non-innervated tissue, safe from clearance by dopamine transporters. It is this pooling effect, a simple but powerful phenomenon, which is ultimately responsible for normalization of dopamine tone across the preclinical denervation range. Originally, "Passive Stabilization" was proposed to breakdown at severe denervation leading to decreased dopaminergic tone, because the distance between dopaminergic terminals is too great for sufficient mixing of released dopamine to generate a steady-state level (Bergstrom and Garris, 2003). Demonstration of pooling in the absence of the second clearance mechanism negates this postulate. Rather, non-dopamine transporters acting on dopamine in non-innervated tissue appear responsible for the eventual drop in dopaminergic tone.

Altered dopamine dynamics in the lesioned striatum

The striatum, severely dopamine depleted to the degree observed in symptomatic Parkinson's disease, exhibits a radical form of dopaminergic neurotransmission characterized by extreme extrasynaptic signaling (Zigmond et al., 1990;Zoli et al., 1998). The loss of dopamine transporters permits released dopamine to diffuse, relatively speaking, great distances (Doucet et al., 1986;van Horne et al., 1992;Schneider et al., 1994). The resulting slow extracellular clearance is expected to prolong what normally are transient increases in dopamine concentration following release, much like those documented in dopamine transporter knockouts (Jones et al., 1998) and following psychomotor stimulant inhibition of dopamine uptake (Venton et al.,

2003). The present simulations provide additional support for altered extracellular dopamine dynamics in the severely lesioned striatum. Once dopamine escapes innervated tissue, release events are prolonged, giving rise to slow, broad changes in the range of extracellular dopamine concentration (Fig. 7).

Although a drop in dopaminergic tone is proposed to lead to cardinal symptoms of Parkinson's disease (Zigmond et al., 1990), it is interesting to speculate that slowed extracellular dopamine dynamics also play a role. Changes in extracellular dopamine concentration, relatively transient, large and frequent in the intact striatum, passivate with progressive denervation. It is unknown whether slowed extracellular dynamics alters dopamine functions such as switching target cells from an off to an on state, rendering them more sensitive to (Patricio Odonel), and modulating long-term potentiation (Calabrese). Emerging evidence indicates that Parkinson's disease is a complex disorder exhibiting motor and cognitive deficits (Kulisevsky, 2000; McNamara et al., 2003). Because the relationship between various symptoms and deficits in nigrostriatal dopaminergic neurotransmission is not completely established, altered extracellular dynamics should be considered in addition to changes in dopaminergic tone.

Conclusions

This study provides a theoretical framework for a new hypothesis of compensatory adaptation during the preclinical phase of Parkinson's disease. In "Passive Stabilization", dopaminergic tone is normalized without active changes in release and uptake. Conserved features of the mammalian nigrostriatal dopaminergic

innervation ( ), and the fact that "Passive Stabilization" is based on the physical principles of steady state and diffusion, suggest that this new compensatory scheme should apply in Parkinson's disease. Because many neurotransmitters diffuse and signal extrasynaptically similar to dopamine (Zoli et al., 1998;Vizi, 2000), "Passive Stabilization" may generalize to other neurodegenerative disorders that are progressive and emerge later in life such as Huntington's and Alzheimer's disease (Bergstrom and Garris, 2003). Simulations also identify a potentially new target for pharmacological intervention in Parkinson's disease, glial catecholamine transporters. By virtue of slow uptake kinetics, inhibiting this transporter will only be effective for increasing dopaminergic tone in severely dopamine-depleted brain regions.

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FIGURE LEGENDS

Figure 1

Figure 1 is the illustration of the old model used for the simulations

Figure 2A

Figure 2A is the plot of concentration (DA) versus time for the different %s of innervations. The different values of innervations used for this simulation are 1, 5, 10, 20, 40, 60, 80, and 100. The total simulation time for each innervation level was 301 seconds during which 669 samples were obtained at regular intervals. The set values obtained for each level of innervations were averaged across 10 different patterns.

Figure 2B

Figure 2B is similar to figure 2A and makes use of the same data for the graphs. The only difference is that this graph plots the values for the first 18 seconds of the simulation.

Figure 3A

Figure 3A is a plot of concentration (DA) versus the different %s of innervations. Values from 10 different patterns were used to plot the graph with error bars. For each pattern the values were obtained by average the DA values over 60 seconds from 300 to 301 seconds, long after the steady state had been reached.

Figure 3B

Figure 3B is a plot of concentration levels across the middle 100 micrometer of the 200 micrometer nerve tissue being modeled in the simulation. The plot is of single pattern and for all levels of innervations. The values were averaged over 60 seconds from 300 to 301 seconds of simulation.

Figure 3C

Figure 3C is a plot of range values for different levels of innervations sampled during the last 6 seconds of the simulation. The sample size is 49 and the values from a single pattern.

Figure 3D

Figure 3D is plot of range values for the different levels of innervations. The range values are obtained for all the levels of innervations from 10 different patterns. For each pattern and level of innervation, the concentration values were averaged over 60 seconds from 300 to 301 seconds of simulation.

Figure 4

Figure 4 is the plot of concentration levels across the middle 100 micrometer of the 200 micrometer nerve tissue being modeled in the simulation and the innervation level is 1%. The plots are made of concentration levels at different intervals of time. The plot indicates how the model reaches the steady state as time progresses.

Figure 5

Figure 5 is the illustration of the new model used for the simulations

Figure 6

Figure 6 is a plot of concentration levels across the middle 100 micrometer of the 200 micrometer nerve tissue being modeled in the simulation. There is a plot for every value of K. For the simulation the following values of k were used – 0.00, 0.01, 0.05, 0.1, 0.25 and 1.0. The values were averaged over 60 seconds from 300 to 301 seconds of simulation and across 10 patterns for each of the value of k.

Figure 7A

Figure 7A is the plot of concentration (DA) versus time for the different %s of innervations. The different values of innervations used for this simulation are 1, 5, 10, 20, 40, 60, 80, and 100. The total simulation time for each innervation level was 301 seconds during which 669 samples were obtained at regular intervals. The set values obtained for each level of innervations were averaged across 10 different patterns and the non specific uptake value was set at 0.1.

Figure 7B

Figure 7B is a plot of concentration levels across the middle 100 micrometer of the 200 micrometer nerve tissue being modeled in the simulation. The plot is of single pattern

and for all levels of innervations and the non specific uptake value was set at 0.1. The values were averaged over 60 seconds from 300 to 301 seconds of simulation.

Figure 7C

Figure 7C is a plot of range values for different levels of innervations sampled during the last 6 seconds of the simulation. The sample size is 49 and the values from a single pattern and the non specific uptake value is set at 0.1.

Figure 7D

Figure 7D is plot of range values for the different levels of innervations. The range values are obtained for all the levels of innervations from 10 different patterns and the non specific uptake value is set at 0.1. For each pattern and level of innervation, the concentration values were averaged over 60 seconds from 300 to 301 seconds of simulation.

Figure 8

Figure 8 is the plot of concentration levels across the middle 100 micrometer of the 200 micrometer nerve tissue being modeled in the simulation. The innervation level is set to 1% and the non specific uptake value was set at 0.1. The plots are made of concentration levels at different intervals of time. The plot indicates how the model reaches the steady state as time progresses.

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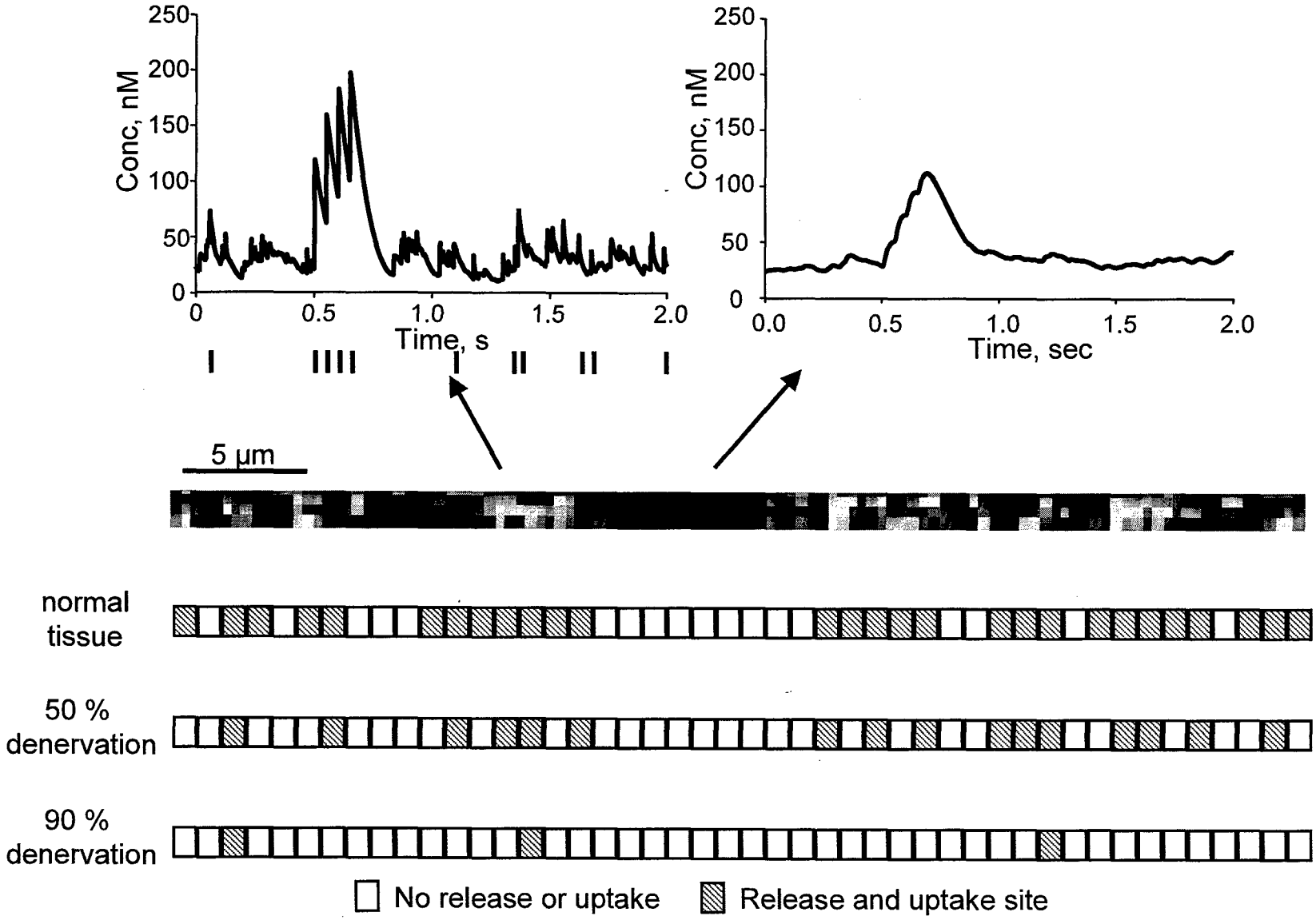
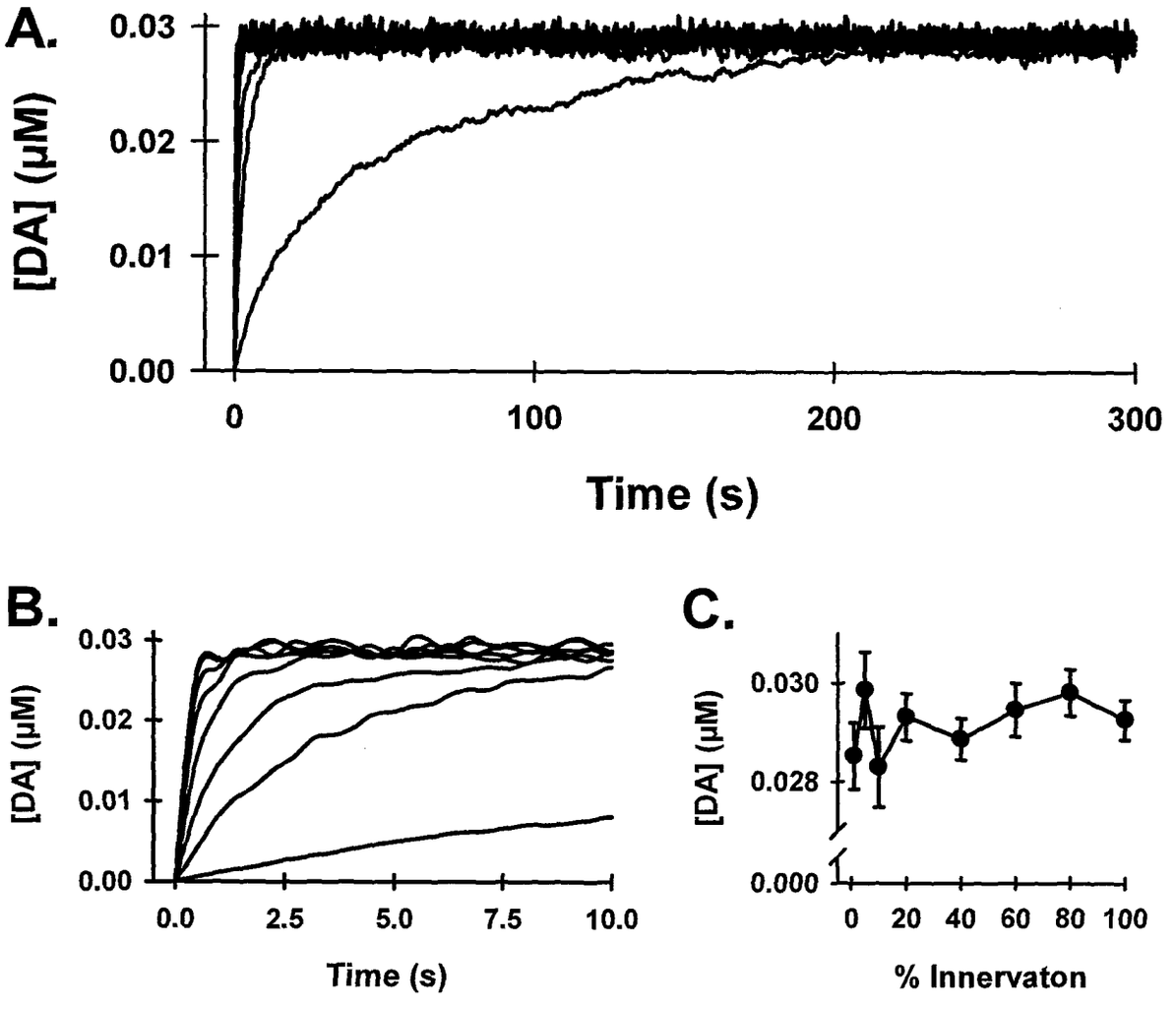


Venton et al. Figure 1



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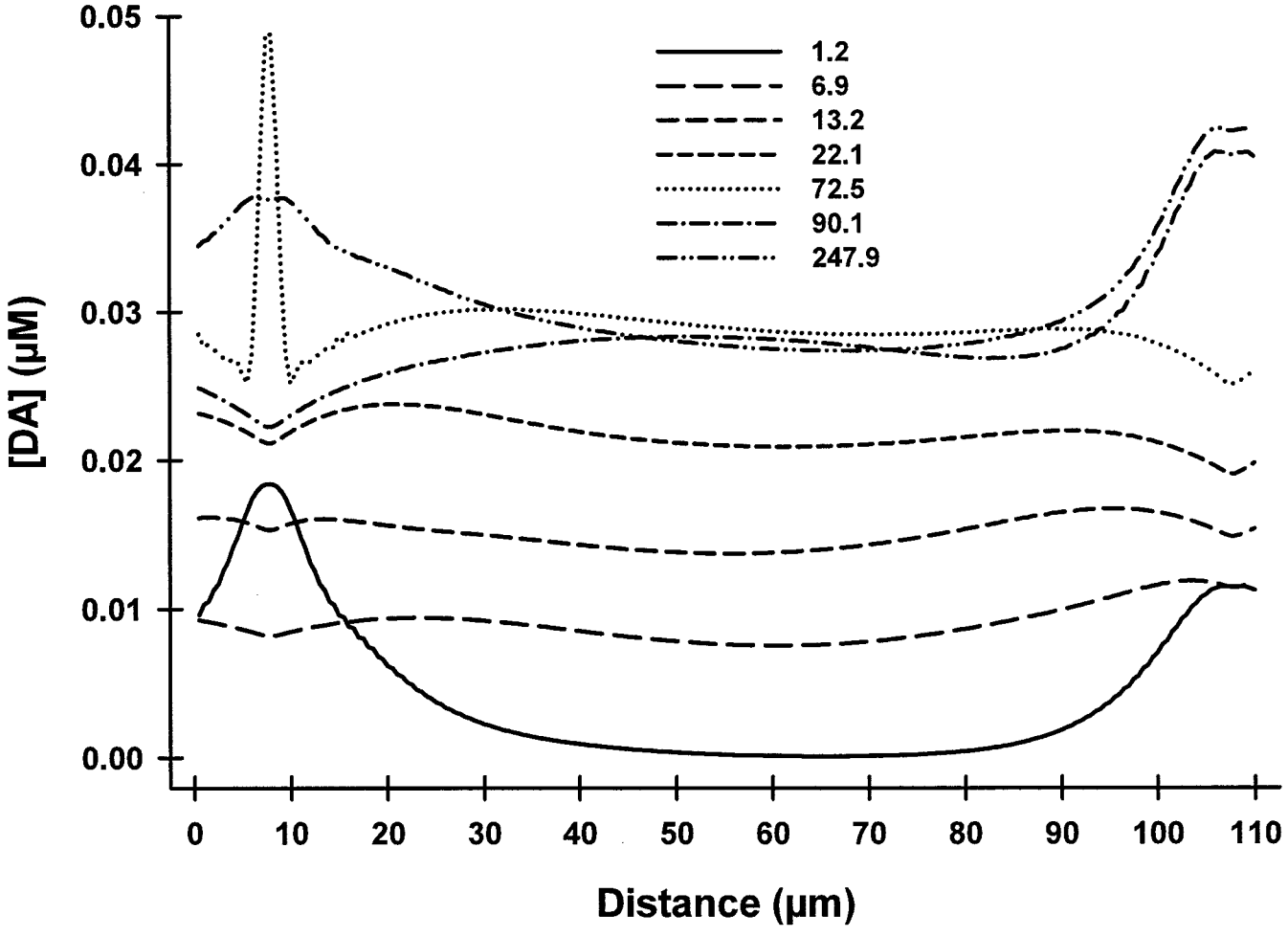

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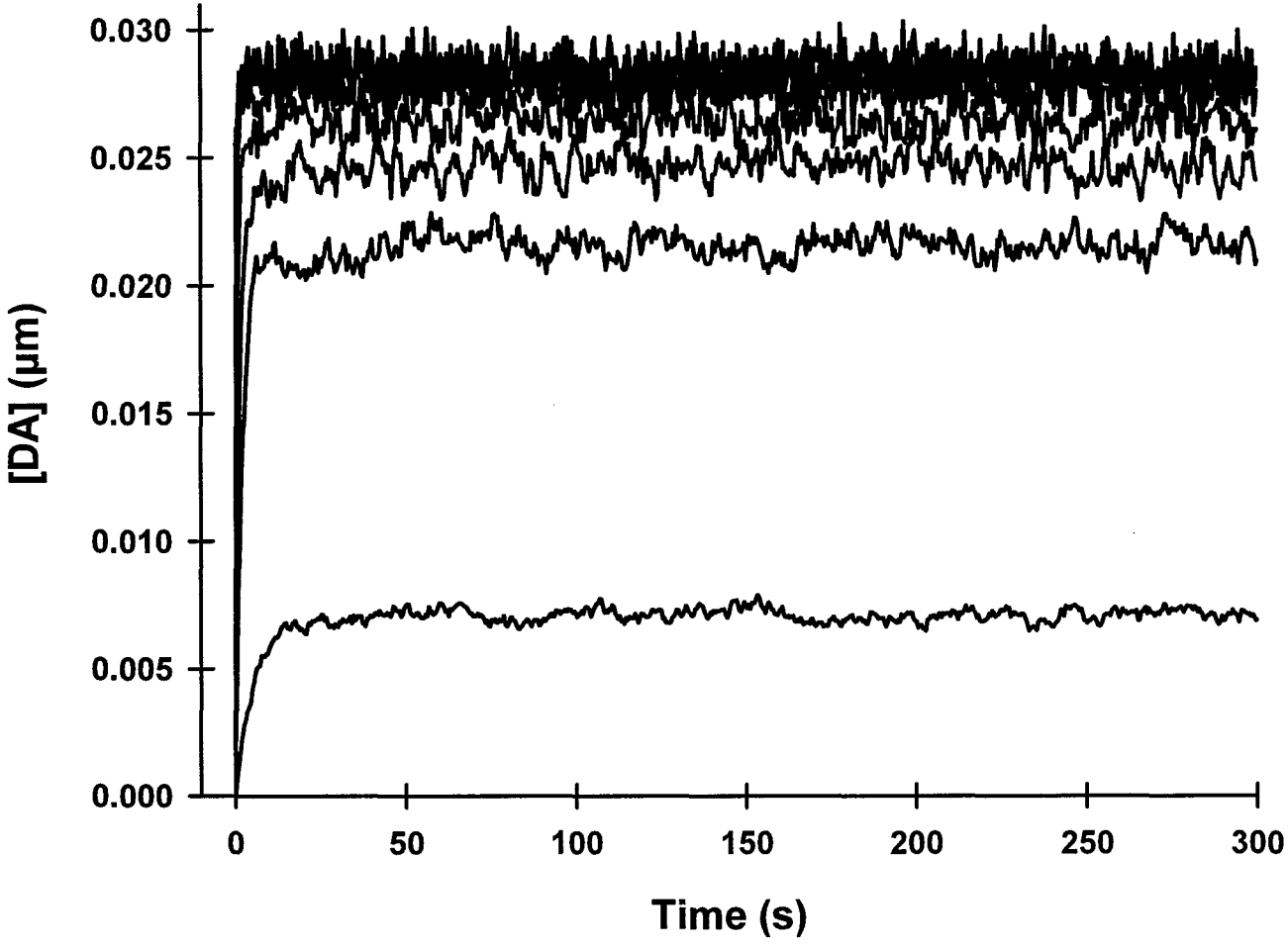
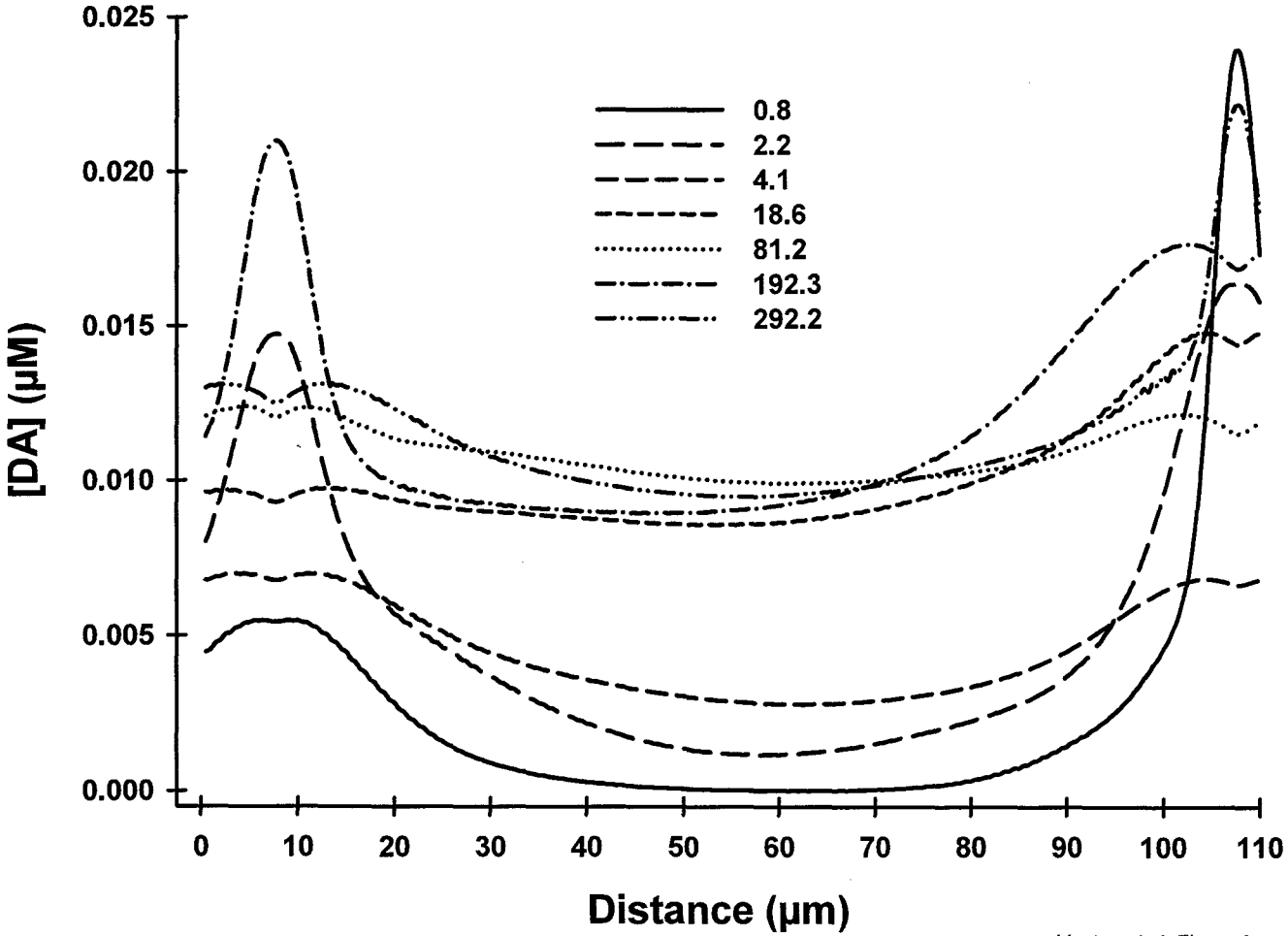


Venton et al. Figure 5



Venton et al. Figure 6

Comparison of "traditional" and "extended" Waveforms used with fast-scan cyclic voltammetry for modeling dopamine release and uptake in the rat striatum

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Fast-scan cyclic voltametry (FSCV) allows for the detection of electroactive neurotransmitters, such as dopamine (DA), with millisecond sampling. This fine temporal resolution permits characterizing extracellular DA dynamics and the release and uptake mechanisms determining these dynamics. Enhanced sensitivity of FSCV has been reported by extended the applied triangle waveform from, e.g., -0.4 to 1.0 V (i.e., " traditional waveform") to -0.6 to 1.4 V (i.e., "extended waveform"). However, the extended waveform increases sensitivity at the expense of response time, and a slowed response time may not support kinetic studies of DA release and uptake. In this study we compare traditional and extended waveforms for modeling DA release and uptake in the striatum of the urethane-anesthetized rat. A stimulating electrode was placed in the median forebrain bundle, and a carbon-fiber microelectrode was implanted in the dorsomedial striatum. The extended waveform increased sensitivity by 10 fold, in agreement with previous work. This increase reached a plateau within 20 min after switching from the traditional to extended waveform and was stabile for at least two hours. Kinetic analysis of the evoked recordings collected by either the traditional or extended waveform demonstrated no significant differences for dopamine uptake, as measured by Vmax (1.85±0.43 and 1.57±0.42 μM/s, respectively), and DA release, as measured by the concentration of DA elicited per stimulus pulse (45±10 and 44±10 nM, respectively). These results demonstrate that kinetic analysis of release and uptake can be successfully performed on electrically evoked DA levels recorded by FSCV using the extended waveform.

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